# EXHIBIT A

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SHORT REPORT

# A novel type of mutation in the cysteine rich domain of the RET receptor causes ligand independent activation

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**Multiple endocrine neoplasia type 2A (MEN 2A) is a dominantly inherited cancer syndrome, which involves the triad of MTC, pheochromocytoma, and hyperparathyridism. Missense mutations in one of six cysteine codons in the extracellular cysteine-rich domain of the *RET* proto-oncogene predispose to this disease. These mutations cause ligand-independent constitutive activation of the tyrosine kinase receptor by the formation of disulfide-bonded homodimers. We examined a different type of mutation, which results in an additional cysteine in the cysteine rich domain. A duplication of 9 bp in the first case resulted in an insertion of three amino acids between codon 633 and 634. In the second case a 12 bp duplication in exon 11 results in four additional amino acids between codon 634 and 635. Here we demonstrate that an additional cysteine causes a ligand independent dimerization of the RET receptor in transfected NIH3T3 cells, which results in an activation of the intracellular tyrosine kinase.** *Oncogene* (2000) **19**, 3445–3448.

**Keywords:** MEN 2; *RET*-proto-oncogene; additional cysteine 634; ligand independent dimerization

The *RET* proto-oncogene codes for a receptor tyrosine kinase which plays a role in the development of the neural crest and its derivates. Besides this, RET is important for kidney development (Pachnis *et al.*, 1993; Avantaggiato *et al.*, 1994). In adult humans RET is expressed in cells of the nervous system, in C-cells, the adrenal medulla and the parathyroids (Nakamura *et al.*, 1994). It has been shown that the glial cell line-derived neurotrophic factor (GDNF), neurturin (NTN) and persephin are functional ligands for the RET receptor (Durbec *et al.*, 1996; Baloh *et al.*, 1997; Milbrandt *et al.*, 1998). Activation of the tyrosine kinase is mediated by glycosylphosphatidylinositol-(GPI)-anchored cell surface RET-coreceptors GDNFR-α1–3 (Treanor *et al.*, 1996; Jing *et al.*, 1996; Baloh *et al.*, 1997).

Mutations in the *RET* proto-oncogene are involved in several inherited and non inherited diseases. Inactivating mutations cause familial and sporadic Hirschsprung disease, a syndrome of congenital absence of enteric innervation (Romeo *et al.*, 1994). Activating mutations have been identified as the underlying cause of multiple endocrine neoplasia Type 2 (MEN 2) and familial medullary thyroid carcinoma (FMTC) (Donis-Keller *et al.*, 1993; Mulligan *et al.*, 1993). Mutations in one of six cysteine codons in exon 10 or exon 11 occur in most cases of MEN 2A and FMTC. These mutations convert *RET* into a dominant transforming oncogene (Santoro *et al.*, 1995). Substitutions of cysteine residues related to the extracellular cysteine-rich domain result in ligand independent dimerization of the receptor and constitutive activation by autophosphorylation of specific tyrosine-residues in the intracellular kinase domain (TK) (Santoro *et al.*, 1995). Probably the Cys residues, disrupted by the MEN 2A mutations, are normally involved in intramolecular disulfide bonds. The mutations may render the partner Cys available for aberrant disulfide binding with other mutant RET or wt-RET molecules and thus form activated dimers. The activated RET binds the adaptor proteins Shc, Grb2 and PLC-γ (Borrello *et al.*, 1994, 1996). Rare MEN 2A/FMTC-associated mutations involve the intracellular kinase domain. In particular the codons 790, 791 and 804 in the TK1 domain are affected (Berndt *et al.*, 1998; Fattoruso *et al.*, 1998).

Recently we described a novel type of MEN 2A associated mutation which results in an additional Cys 634 in exon 11. In the germline of a patient with the complete phenotype of MEN 2A a duplication of 9 bp representing the codons 634, 635 and 636 was found (Höppner *et al.*, 1998). In the second case a 12 bp-duplication which results in additional four amino acids between codon 634 and 635 was detected in a family with 14 affected members (Höppner *et al.*, 1997). While the 9 bp-duplication is in frame, the inserted 12 bp sequence is not in frame which results in an additional histidine residue at the 5′ end of the breakpoint. The 12 bp duplication is associated with a different MEN 2A phenotype. Besides MTC an unusual accumulation of pHPT occurs, none of the 14 affected family members have pheochromocytomas (Höppner *et al.*, 1997).

To examine the mechanism of activation of the mutated RET, we cloned the *RET*-cDNA of the short isoform into the eucaryotic expression vector pRC/CMV2 and inserted the mutations described above by fragmental cloning in the case of the 9 bp-duplication (+9 bp-RET) and by PCR-mutagenesis for the 12 bp-duplication (+12 bp-RET). The mouse fibroblast cell line NIH3T3 was transfected and clones expressing wt-RET, +9 bp-RET and +12 bp-RET were selected. TT-cells, a human medullary thyroid carcinoma cell line which is heterozygous for one of the classical mutations in codon 634 (C634W) of the *RET* proto-oncogene was also cultured. After cell lysis we analysed the proteins by nonreducing and reducing SDS-polyacrylamid gel electrophoresis (Figure 1).

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### Ligand independent dimerization of the +9 bp- and +12 bp-RET

In agreement with previous reports (Santoro *et al.*, 1995) the mutation C634W results in ligand independent dimerization of the RET receptor, which is represented by the 300 kDa band of RET expressed in TT-cells (Figure 1a, lane 1). The monomeric forms migrate as 150 and 170 kDa bands. The +9 bp-RET (Figure 1a, lane 2) and +12 bp-RET (Figure 1a, lane 3) also migrate as dimers whereas the wt-expressing cells only show the monomeric RET (Figure 1a, lane 4). Therefore it can be concluded that the additional Cys 634 results in a ligand independent dimerization of the RET receptor.

### Formation of RET-dimers by intermolecular disulfide linkages

As shown in previous studies (Santoro *et al.*, 1995), under reducing conditions the C634W-RET migrates as a monomer (Figure 1b, lane 1). This serves as an evidence that the mutation causes a ligand independent dimerization by formation of intermolecular disulfide linkages. Under reducing conditions also the +9 bp-RET (Figure 1b, lane 2) and the +12 bp gene product (Figure 1b, lane 3) are only detectable as monomeric forms. Obviously the additional Cys 634 also causes interchain disulfide linkages which result in ligand independent dimerization.

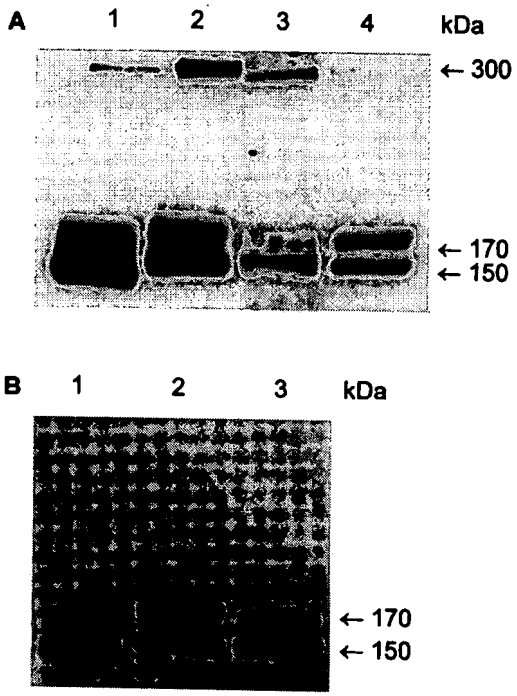


**Figure 1** (a) Western blot analysis of wt and mutated RET receptors under nonreducing conditions. Comparable amounts of total cellular proteins from TT-cells (lane 1), and NIH3T3 cells stable transfected with +9 bp-RET (lane 2), +12 bp-RET (lane 3) and wt-RET (lane 4) were dissolved in loading buffer without DTT and subjected to SDS–PAGE. The proteins were detected after Western blotting with a polyclonal anti-RET antibody (Santa Cruz). Immunocomplexes were visualized by enhanced chemiluminescence detection kit (ECL, Pierce) using anti-goat coupled to horseradish peroxidase (DIANOVA). +9 bp-RET, +12 bp-RET and C634W-RET (lane 1) migrate as dimeric forms represented by the band at 300 kDa while the wt-RET migrates only as monomeric forms at 150 and 170 kDa. (b) Under reducing conditions +9 bp-RET gene product (lane 2) and the +12 bp protein (lane 3) show a molecular weight of 150 and 170 kDa. The positive control, C634W-RET (lane 1) also migrates as monomers

### Constitutive activation of the +9 bp- and +12 bp-RET tyrosine kinase in NIH3T3 cells

To investigate the ligand independent activation of the mutated receptors, RET was immunoprecipitated from the various NIH3T3 transfectants and assayed for phosphotyrosine (pTyr) content, a hallmark of receptor activation. In case of the TT cells we could confirm previous results (Santoro *et al.*, 1995) which show that the mutation C634W causes an autophosphorylation of the intracellular tyrosine kinase (Figure 2b, lane 1). This also has been shown for RET receptors with Cys 634 substitution expressed in NIH3T3 cells. Our results demonstrate that the +9 bp- and +12 bp-RET proteins also are autophosphorylated (Figure 2b, lane 2 and lane 3). In comparison with the TT-cells there is a similar amount of pTyr in the mutated receptors with the additional cysteine 634. These results show that this type of mutation causes a constitutive activation of the receptor tyrosine kinase after dimerization.

One could speculate that overexpression of the wt-*RET* is sufficient to trigger the kinase activity. To determine whether overexpression or the additional cysteine 634 causes the constitutive activation, we compared cells with less expression of the +12 bp-

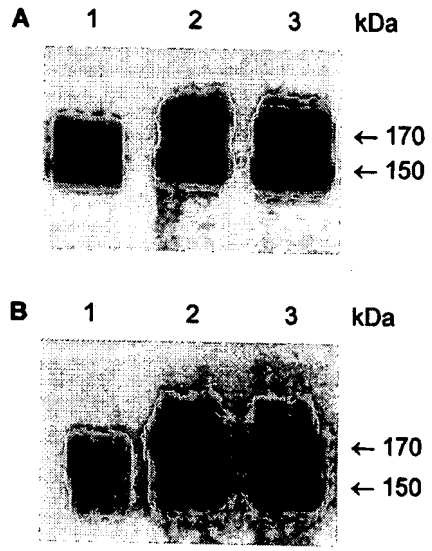


**Figure 2** Activation of the tyrosine kinase of the RET receptor. Comparable amounts of total cellular proteins from the indicated transfectants were immunoprecipitated with a polyclonal anti-RET antibody. The immunoprecipitates were then either immunblotted with anti-RET (**a**) or a monoclonal antibody to phosphotyrosine (pTyr PY99) (Santa Cruz) (**b**). In case of the duplication in exon 11 the *RET* product shows high amounts of pTyr (**b**: lanes 2 and 3). The RET protein of the TT-cells (**a**: lane 1) shows a low signal for pTyr (**b**: lane 1) but in relation to the expression level it is comparable with the +9 bp-RET and the +12 bp-RET

RET and cells with a higher expression of wt-RET. The wt-RET displays autophosphorylation but in comparison with the +12 bp-RET the amount of pTyr is significantly less (data not shown). This demonstrates that it is not the level of expression that causes ligand independent activation but the mutation itself.

### Grb2-binding by the activated RET

It has been shown that activated RET receptors bind Grb2 and trigger intracellular signal pathways. To confirm that receptor activation mediated by an additional Cys 634 leads to Grb2 binding we performed coprecipitation experiments of Grb2 and the RET protein. Our results show that the receptors with the duplications in exon 11 bind Grb2 (Figure 3b, lane 2 and lane 3) as is the case for the RET protein with the classical mutation in codon 634 (Figure 3b, lane 1). This is also the case for the +12 bp-RET clones with low expression (Figure 3b, lane 3).

These data present evidence for receptor activation by a duplication of a sequence including the critical cysteine residue in codon 634. *In vitro* no differences in the mechanism of activation occur between the 9 bp- and the 12 bp-duplication. The phenotype of the +12 bp-transfectants differs from that of the +9 bp-transfectants (data not shown). The latter show the complete transformed phenotype with cell clusters and spindle shape whereas the +12 bp-transfectants only express cell clusters but exhibit the same cell shape as wt-transfected cells. Interestingly the *in vivo* phenotype of the +12 bp-family differs from that of families with the classical mutations and the +9 bp-patient. The fact that no pheochromocytoma occurs but that pHPT does suggest that tissue specific mechanisms exist.

The different mutations investigated in this study alter significantly the chemical properties of the cysteine-rich domain in close vicinity to the plasma membrane and lead to considerably different spacing of the cysteine codons. In contrast to the missense mutation in codon 634 the two duplication mutations leave the first extracellular cysteine at the same distance from the cell surface as in the wt-RET (Figure 4). Also the recently published deletion of six base pairs (Bongarzone *et al.*, 1999), which represents an activating mutation too, still leaves the cysteine 634 in place (Figure 4). Thus the distance of the cysteine relative to the plasma membrane seems not to be essential.

It has not been shown whether Cys 634 and Cys 630 are involved in intrachain disulfide bonds *in vivo*. The fact that substitutions and insertions of Cys 634, just as deletions between Cys 634 and Cys 630, result in ligand independent dimerization suggest an essential function of this domain in transition from inactive monomers to activated dimers. The distance between Cys 634 and the next extracellular cysteine in codon 630 is two amino acids in the Δ6-RET, three amino acids in the +9 bp-RET and four amino acids in the +12 bp-RET and the wt-RET receptor respectively (Figure 4). If intrachain disulfide bonds should form between these cysteines, the energy of the bonds may differ significantly or may even become sterically impossible. In the case of cysteine duplication, there may be a competition between disulfide linkage of the new cysteine with cysteine 634 and 630. For the 12 bp duplication, the distance in both directions is the same and equals the wild type conditions. The only difference is an Asp–His exchange in the three amino acids between the cysteines. In this case disulfide linkages may be equally likely to both sides. Thus

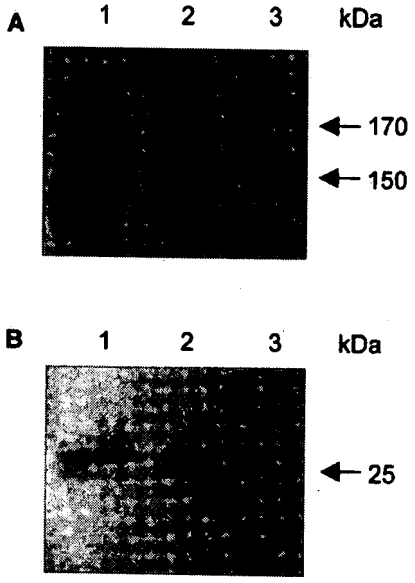


**Figure 3** Grb2-binding of the mutated RET. Comparable amounts of total cellular proteins from the indicated transfectants were immunoprecipitated with an anti-RET antibody. The immunoprecipitates were then either immunoblotted with anti-RET (a) or antiGrb2 (Dianova) (b). Although the +12 bp-RET shows a low expression (a: lane 3) in comparison with the RET protein expressed in TT-cells (a: lane 1) and the +9 bp-RET (a: lane 2), the amount of coprecipitated Grb2 is comparable (b: lane 3)

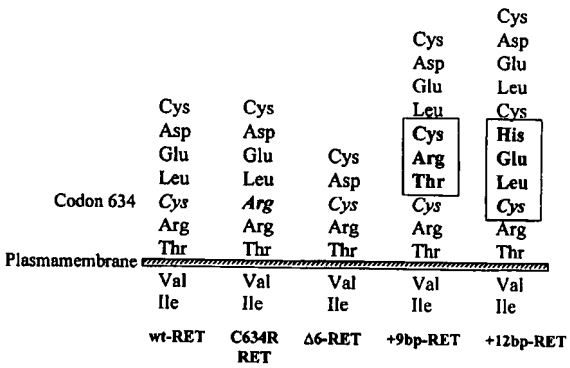


**Figure 4** Schematic illustration of the amino acid sequence in the vicinity of the plasma membrane. The wt-sequence shows the original arrangement of Cys 630 and Cys 634. Four types of mutation are described which affect this domain. The classical mutation is a substitution of Cys 634. In this figure the mutation Cys 634Arg is shown, but other amino acid changes are described. Another type of mutation is a 6 bp-deletion (Δ6) which does not affect Cys 634 directly, but moves Cys 630 and Cys 634 closer to one another. The type of mutation described in this paper is a duplication of Cys 634 (+9 bp and +12 bp). In the case of the 9 bp-duplication, the sequence Thr-Arg-Cys is inserted (box), the 12 bp-duplication results in the additional sequence Cys-Leu-Glu-His (box)

either cysteine 630 or 634 may be available for interchain disulfide linkage.

For the 9 bp duplication the distance to cysteine 630 is four codons and to cysteine 634 is three codons. The disulfide linkage between cysteine 630 and the new cysteine would be the same as in the wild type and may be preferred, one cysteine remaining free and this may become involved in interchain disulfide linkage. For the Δ6 bp-*RET* the distance of the two cysteines may be too small to facilitate intrachain disulfide linkage (Bongarzone *et al.*, 1999).

It is not certain whether intrachain disulfide bonds occur between Cys 630 and Cys 634. Another possibility is that these residues form bridges with more distant cysteine residues. In this case it is possible that displacement occurs between the inserted cysteine and the original Cys 634. The different pattern of disulfide bonds would alter the stability of interchain disulfide bridges for the 12 bp-duplication.

In summary, the duplication of Cys 634, similar to the substitution in the classical MEN 2A associated mutations, results in an alteration of the number of cysteines which results in spontaneous receptor dimerization. In addition, the different spacing of cysteines may modulate the transformational capacity in various tissues resulting in a different clinical phenotype.

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**EXHIBIT B**

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# The Glu632-Leu633 deletion in cysteine rich domain of Ret induces constitutive dimerization and alters the processing of the receptor protein

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**Mutations of the *RET* gene, encoding a receptor tyrosine kinase, have been associated with the inherited cancer syndromes MEN 2A and MEN 2B. They have also further been associated with both familial and sporadic medullary thyroid carcinomas. Missense mutations affecting cysteine residues within the extracellular domain of the receptor causes constitutive tyrosine kinase activation through the formation of disulfide-bonded homodimers. We have recently reported that a somatic 6 bp in-frame deletion, originally coding for Glu632-Leu633, potently activates the *RET* gene. This activation is increased with respect to the frequent MEN 2A-associated missense mutation Cys634Arg. This finding specifically correlated to the clinic behavior of the corresponding tumor, which was characterized by an unusually aggressive progression with both multiple and recurrent metastases. By examining the possibility that this deletion acts in a manner similar to cysteine substitution, we have analysed the molecular mechanism by which this oncogenic activation occurs. Phosphorylated dimers of the deleted Ret receptor were detected in immunoprecipitates separated under non-reducing conditions. Like other Cys point mutations, this 6 bp deletion affecting two amino acid residues between two adjacent Cys, is capable of activating the transforming ability of Ret by promoting receptor dimerization. These results suggest that alteration to cysteine residue position or pairing is capable of inducing ligand independent dimerization. Furthermore, we present data demonstrating that the processing and sorting of the Ret membrane receptor to the cell surface is affected by mutation type.**

**Keywords:** *RET*; oncogenic activation; dimerization

## Introduction

Medullary thyroid carcinoma (MTC) is a malignant tumor arising from calcitonin-secreting parafollicular C cells. MTCs may occur sporadically or as a component of the familial cancer syndrome known as multiple endocrine neoplasia type 2 (MEN 2A). Germline mutations within the *RET* proto-oncogene, coding for a receptor tyrosine kinase, can cause the MEN 2A, MEN 2B, and FMTC syndromes. The majority of germline mutations reportedly associated with MEN 2A and FMTC lie within exons 10 and 11 of *RET*. These mutations specifically occur at codons 609, 611, 618, 620 and 634 and universally result in the substitution of a cysteine residue (Mulligan *et al.*, 1993; Donis-Keller *et al.*, 1993). A single point mutation at codon 918, causing the substitution of a methionine (ATG) with a threonine (ACG), is associated with both MEN 2B and sporadic MTC (Eng *et al.*, 1994, 1995b; Carlson *et al.*, 1994; Hofstra *et al.*, 1994; Muragaki *et al.*, 1995; Blaugrund *et al.*, 1994). Approximately 80% of sporadic MTCs are positive for this specific *RET* codon 918 mutation (Eng *et al.*, 1995b, 1996). In contrast, two other relatively rare somatic mutations, at codons 768 (exon 13) and 883 (exon 15), are detected in approximately 10% of sporadic MTCs (Bolino *et al.*, 1995; Eng *et al.*, 1995a; Komminoth *et al.*, 1995). We have recently described two additional interstitial deletions affecting exons 10 and 11 (Romei *et al.*, 1996; Ceccherini *et al.*, 1997). These novel somatic mutations, found in two different patients, were heterozygous in frame deletions of 48 (cod 592–607) and 6 bp (cod 632–633) respectively, yet did not directly involve any cysteine residues. Other similar codon deletions have been observed in sporadic MTCs, namely a 3 bp deletion (cod 633) (Hofstra *et al.*, 1996), and a 27 bp deletion comprising exon 10 (Kalinin and Frilling, 1998). Of interest, a duplication of 12 bp between codon Cys634 and Arg635, resulting in the insertion of four amino acids including a cysteine, has been described as a novel MEN 2A family germline mutation (Hoppner and Ritter, 1997). It has been demonstrated that missense mutations involving any of the cysteine residues 609, 611, 618, 620 and 634, typical of the MEN 2A and FMTC phenotypes yet rarely identified in sporadic MTCs, can promote ligand independent Ret dimerization leading to constitutive enzymatic activation. It is further evident that the substitution of one of these five crucial cysteines leads to both ligand independent dimerization and receptor phosphorylation. We have recently demonstrated in an *in vitro* system that the deletion of the codons 632–633 more effectively activated the *RET* gene with respect to the Cys634Arg missense mutation. This specifically correlated to the clinical severity of the corresponding tumor, characterized by an unusual aggressive progression with both multiple and recurrent metastases (Ceccherini *et al.*, 1997).

In this paper, we addressed the problem of the molecular mechanism underlying this oncogenic activation. We examined the possibility that these deletions act in a similar way to Cys point mutations. Phosphorylated dimers of the deleted Ret receptor

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are detected when immunoprecipitates are separated under non-reducing conditions. Therefore, like Cys point mutations, this deletion affecting two Cys-proximal residues is capable of activating the transforming ability of Ret by promoting ligand-independent receptor dimerization.

Furthermore, we have studied the biogenesis of RetCys634Arg and Retdel1632–633 and found that the latter to be more slowly processed to the cell surface. A large fraction of Retdel632–633 intermediates and a significantly smaller fraction of RetCys634Arg, is retained within the endoplasmic reticulum where it dimerizes and transduces mitogenic signals.

## Results

### *Effect of Δ6 deletion mutation on dimerization and autophosphorylation of Ret receptor*

Unlabeled lysates from Retdel632–633 (RetΔ6), RetCys634Arg (Ret2A) and Ret wild-type (Retwt) expressing cell lines were immunoprecipitated with an anti-Ret antibody. Immunocomplexes were tested for kinase activity in the presence of [$\gamma$-$^{32}$P]ATP. A significant amount of Ret autophosphorylation was seen in the cell lines expressing the RetΔ6 and Ret2A constructs but not in the wild-type (data not shown). This was demonstrated by the labeling of two proteins corresponding to the Ret isoforms of approximately 140 and 160 kDa. Therefore, even in the absence of ligands, both RetΔ6 and Ret2A can be phosphorylated.

To examine whether increased kinase activity correlated with increases in covalent dimer formation, we analysed immunoprecipitated RetΔ6, Ret2A and Retwt proteins by SDS–PAGE under both reducing and non-reducing conditions. As shown in Figure 1A, Ret immunoprecipitates separated under non-reducing conditions revealed the presence of Ret dimers in cell lines expressing mutant receptors. Stable dimer formation was not detected in a cell line expressing the wild-type Ret receptor. Furthermore, RetΔ6 and Ret2A dimers were shown to be strongly phosphorylated (Figure 1B) by anti-phosphotyrosine immunoblotting. Two high molecular bands corresponding to the dimerized form of the mature (160 kDa) and the partially glycosylated precursor (140 kDa) of the mutated receptor, appeared phosphorylated. Duplicate immunoprecipitates were subjected to immunoblot analysis for Ret after separation under reducing conditions to determine whether receptor dimerization required disulfide bonding (Figure 1C). Results revealed that under reducing conditions, only monomeric forms of Ret were detected. These results provide evidence that the Ret Glu632-Leu633 deletion promotes disulfide-linked dimer formation. Ret proteins were further analysed under non-reducing conditions by $^{125}$I-labeled protein-A binding (data not shown). Densitometric analysis of bands corresponding to dimers or monomers of the two mutant receptors revealed that RetΔ6 protein exhibits a greater ability to dimerize in comparison to Ret2A protein. The molar ratio of dimers to monomers for RetΔ6 and Ret2A was 0.95 and 0.70 respectively. In contrast, the Retwt control ratio was 0.05. Results from *in vitro* kinase assays, and from both reducing and non-reducing immunoblots demonstrated that the Δ6 deletion mutation is a gain-of-function mutation. This results in the constitutive activation of Ret, thus allowing for ligand-independent signal transduction.

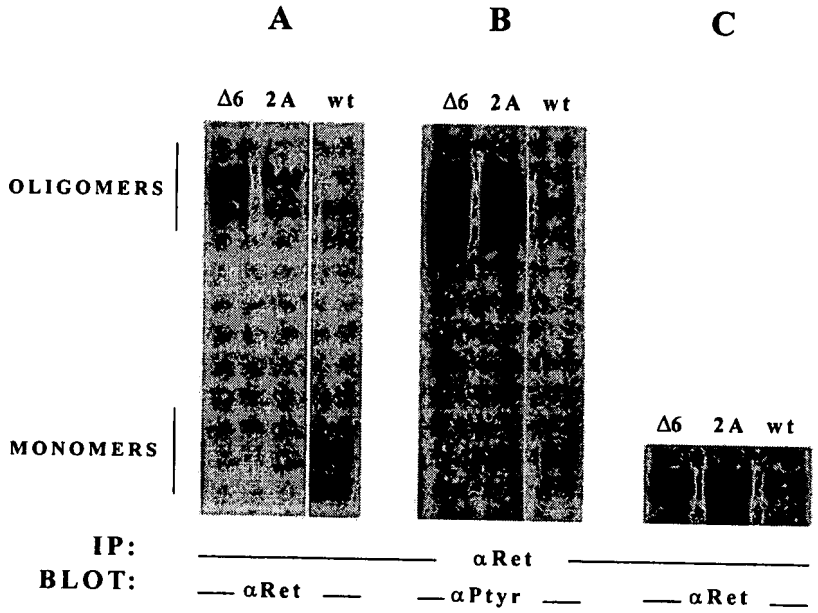


**Figure 1** Dimerization and tyrosine phosphorylation of Ret receptors. Lysates from NIH3T3 cells transfected with the RetΔ6 (lanes Δ6), Ret2A (lanes 2A) and Retwt (lanes wt) constructs were immunoprecipitated (IP) with anti-Ret antiserum (αRet) and the resulting complexes were separated by SDS–PAGE (6%) under non-reducing (**A** and **B**) or reducing conditions (**C**). Western blot filters were developed (BLOT) with anti-Ret (αRet, **A** and **C**) or anti-phosphotyrosine (αPtyr, **B**) antiserum. The positions of the Ret oligomers and monomers are indicated

*Effect of 2-mercaptoethanol treatment on Ret mutants transforming activity*

Reducing agents have previously been used to interfere with the signaling from activated forms of both the thrombopoietin and NEU receptor (Alexander *et al.*, 1995; Siegel and Muller, 1996). We examined whether the addition of 2-mercaptoethanol (2-ME) could interfere with the ability of the RetΔ6 deletion mutant or the Ret2A point mutant to transform NIH3T3 cells. Addition of increasing concentrations of 2-ME (Table 1) failed to affect the transforming activity of the RetΔ6 construct. In contrast, Ret2A transforming activity decreased by 84% at the maximal dose (500 μM) of 2-ME. Toxic effects due to 2-ME at 500 μM were not evident as demonstrated by the unaffected ability of RetΔ6 and HRAS in transforming NIH3T3 cells. This data demonstrates that RetΔ6 has a greater resistance to a reducing agent in comparison with Ret2A. This specifically correlates to a stronger disulfide bond formation in the RetΔ6 mutant.

*Biosynthesis and glycan processing of RetΔ6 and Ret2A proteins and effects of 2-mercaptoethanol treatment*

It has previously been demonstrated that proteins exposing reactive thiols are often retained within the endoplasmic reticulum (ER) (Isidoro *et al.*, 1996). This protein quality control system has been termed thiol-mediated retention. This mechanism is believed to monitor the oxidation status of one or more cysteines contained in protein sequences. Since RetΔ6 and Ret2A mutations affect critical cysteine residues, we studied both the biosynthesis and relative kinetics of dimerization by following the fate of metabolically labeled mutant receptors in NIH3T3 cells. Sub-confluent cultures were labeled with 0.35 mCi/ml $^{35}$S-methionine-cysteine for 15 min and subsequently chased for varying times. Under reducing conditions, newly synthesized protein for both mutant receptors appeared in the 140 kDa form immediately after the pulse (Figure 2). The amount of Ret2A p140 protein increased significantly within the first hour of chase and gradually decreased after 2 h. The mature p160 form appeared after 1 h of chase. In contrast, RetΔ6 p140 reached a maximum intensity only after 2 h of chase, the same chase time at which the mature p160 form became evident. Since the 140 kDa is the endoglycosidase H sensitive form (data not shown and (Asai *et al.*, 1995)) whilst the 160 kDa is resistant, we deduced the longer persistence of the RetΔ6 140 kDa precursor. This indicated that RetΔ6 has a less efficient ER processing compared to the Ret2A precursor. The amount of the mature 160 kDa Ret2A protein was similar to that of the precursor after 2 h of chase, with a maximum reached after 4 h. In contrast, the amount of the 160 kDa RetΔ6 protein increased slowly, yet always remained less than the 140 kDa form. To explore the mechanism of dimerization, features of the labeled RetΔ6 and Ret2A proteins were also followed as a function of time by non-reducing SDS–PAGE (Figure 2). Both the precursor and mature forms of RetΔ6 and Ret2A were capable of dimerization, each with individual specific kinetics (Figure 2). The formation of RetΔ6 homodimers in both the p140 and p160 forms was observed from 2 h. In contrast, after 1 h of chase in the Ret2A mutant, a consistent amount of p140 homodimers and only a small fraction of p160 homodimers were observed. Furthermore, p160 homodimers were observed almost exclusively after longer chase times. Analysis of phosphorylation levels in all RetΔ6 and Ret2A proteins indicated that most of the precursors were significantly phosphorylated, irrespective of whether in the monomeric or dimeric form (Figures 3B and 1B and data not shown). This is due to the fact that phosphorylation associated to dimer forms is intermolecular. Therefore, SDS–PAGE under reducing conditions produces phosphorylated monomers.

**Table 1** 2-mercaptoethanol effect on the transforming activity of Ret mutants

| *Transfected plasmid* (125 ng of DNA) | *Transformed foci/plate at different concentration of 2-ME (μM)* 0 | 30 | 125 | 500 |
|---|---|---|---|---|
| pRCRETΔ6 | 179±11 | 178±7 | 185±26 | 180±19 |
| pRCRET2A | 87±6 | 87±9 | 89±11 | 14±7 |
| pRCRETwt | – | – | – | – |
| pHRAS | 152±18 | 172±11 | 179±7 | 160±4 |

Focus assays were performed with 125 ng of plasmid per plate in the presence of increasing concentrations of 2-ME. Foci were scored over six plates for each concentration after 3 weeks. The HRAS oncogene mutated at its 12th codon was used as a control. Values listed represent the average±s.d. of the number of foci counted on six plates

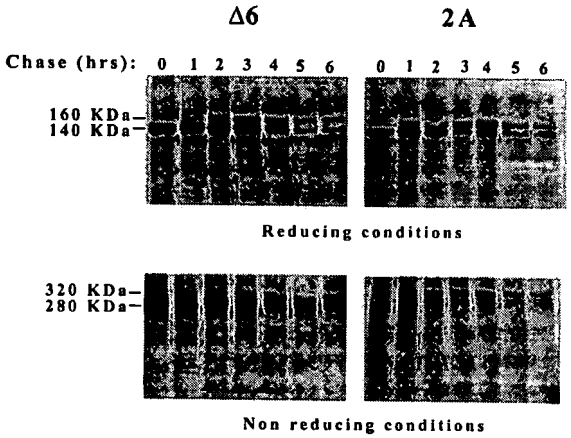


**Figure 2** Pulse-chase labeling of RetΔ6 and Ret2A proteins in reducing and non-reducing conditions. Autoradiograms of metabolically labeled radioactive RetΔ6 and Ret2A proteins derived from transfectants pulsed for 15 min and harvested immediately (lanes marked 0) or after the indicated chase time (lanes marked 1–6). Cell extracts were immunoprecipitated with anti-Ret antiserum and the complexes were separated by SDS–PAGE under either reducing or non-reducing conditions. Gels were exposed to X-ray film for 4–5 days. Numbers are molecular weights in kDa

*Co-immunoprecipitation of SHC phosphorylated proteins with both the precursor and mature forms of the RetΔ6 and Ret2A receptors*

RetΔ6 proteins are characterized by slower maturation kinetics yet display stronger oncogenic activity in comparison to Ret2A proteins. With the aim to explain this fact, we speculated that both the p140 precursor and p160 mature forms of Ret, when

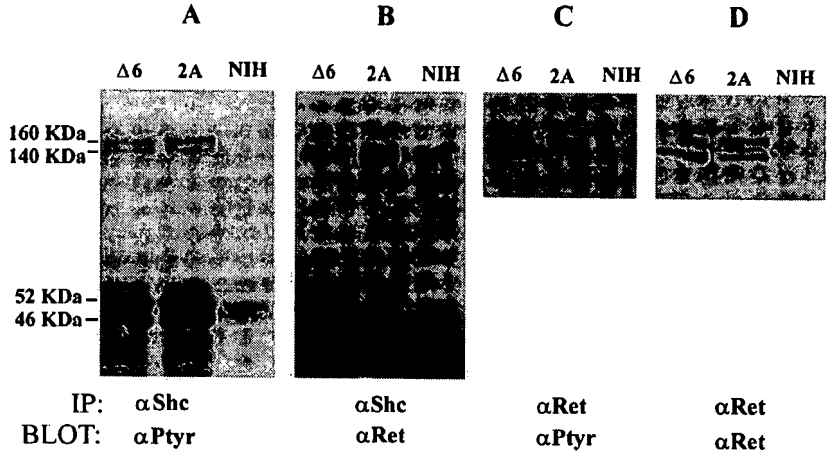


**Figure 3** Coimmunoprecipitation of Shc phosphorylation proteins with both the precursor and the mature forms of RetΔ6 and Ret2A receptors. Lysates from NIH3T3 cells (lane NIH) or from NIH3T3 cell lines expressing the RetΔ6 (lane Δ6) and Ret2A (lane 2A) proteins were immunoprecipitated (IP) with anti-Shc (αShc, **A** and **B**) or anti-Ret (αRet, **C** and **D**) antiserum. The resulting complexes were electrophoresed on a 7.5% SDS–PAGE under reducing conditions. Proteins were transferred to nitrocellulose and subjected to immunoblot analysis (BLOT) with anti-phosphotyrosine (αPtyr) or antiRet (αRet) antiserum. RetΔ6 and Ret2A expressing cell lines were serum starved for 48 h. Numbers are molecular weights in kDa and correspond to two of the three different isoforms of Shc (p52 and p46) and to Ret (p160 and p140), respectively

phosphorylated and dimerized, are able to transduce mitogenic activity in spite of their different localization; the endoplasmic reticulum or surface membrane respectively. To address this, we investigated whether the precursor forms of RetΔ6 and Ret2A were able to bind the Shc adapter protein. This protein is localized to the membrane of the rough endoplasmic reticulum and has been previously demonstrated to bind Ret/PTCs, Ret2A and Ret2B (a mutant Ret receptor carrying an M918T mutation) oncoproteins (Lotti *et al.*, 1996). Cell lysates from RetΔ6 and Ret2A transfected NIH3T3 cells were immunoprecipitated with anti-Shc or anti-Ret antibodies and subsequently blotted with anti-Ptyr antibodies (Figure 3). Both the precursor and mature forms of Ret were co-immunoprecipitated with Shc phosphorylated proteins. The band density of both RetΔ6 and Ret2A co-immunoprecipitated or not with Shc protein and probed with anti-Ptyr were checked by densitometric analysis. The observed affinity of Shc for the precursor and mature forms of both the mutated receptors is similar. Considering the sum of the relative amounts, it was calculated that 40% of RetΔ6 and 20% of Ret2A were co-immunoprecipitated with Shc phosphorylated proteins. It is thus possible to conclude from this data that the mitogenic signal transduced by Shc does not depend on maturation or localization of the mutated receptor, but possibly influenced by the specific type of activating mutation.

## Discussion

We have demonstrated that deletion of the residues Glu632-Leu633 of Ret induces stable receptor dimer formation in the absence of ligand. This suggests that constitutive dimerization and autophosphorylation of the receptor protein underlies the potent transforming activity of this Ret mutant. MEN 2A mutations destroy Cys residues, thus indicating that their mechanism of action involves the creation of ligand-independent dimers most likely through aberrant intermolecular disulfide-bond formation. Whilst not directly involving Cys residues, the Δ6 mutation also induces such dimerization. This deletion mutation may indirectly affect neighboring Cys residues by disrupting the normal and most likely intramolecular cysteine pairing, which occurs in the non-activated receptor. This thus leaves the possibility that at least one unpaired cysteine residue may participate in an intermolecular binding event with another altered receptor, resulting in a disulfide-bonded receptor dimer leading to aberrant receptor activation. In fact, a similar mechanism has been proposed to account for the oncogenic activation in Neu deletion mutants. Deletion of conserved cysteine residues in the juxtatransmembrane region of Neu resulted in elevated transforming activities compared to that of the C647S point mutation form (Siege and Muller, 1996). One potential explanation for this observation is that the spatial arrangement between the remaining cysteine residues may play a crucial role in mediating receptor dimerization. For example, certain deletions may result in a free cysteine being exposed at the surface of the receptor, thus making it more accessible to intermolecular disulfide bond formation.

Interestingly, the transforming activity of Ret2A receptor is decreased following the addition of a reducing agent. In contrast, the transforming activity of the RetΔ6 deletion mutant is not significantly impaired at the same non toxic concentration of 2-ME. This may be explained by the formation of more stable RetΔ6 disulfide-linked dimers, thus generating a more efficient covalent dimerization. Alternatively, it is possible that the loss of residues near cysteine 634 results in an unfolding of the cysteine rich domain that may lead to a less compact and more flexible conformation. Such an unfolding could increase the probability of receptor interaction on the plasma membrane, leading to the tyrosine kinase activation

of a greater number of mutant receptors compared to the Ret2A type receptor. Finally, the lower sensitivity of the Δ6 mutant to reducing agents could also be explained by its particular molecular equilibrium shift from monomeric to dimeric/oligomeric forms.

Taken together, these observations suggest that both the number and spatial arrangement of cysteine residues may induce a ligand independent dimerization.

More generally, it is possible that the loss, gain or mutation or residues within the cysteine-rich region of Ret has the capacity to induce stabilization of the dimeric conformation through intermolecular disulfide bonds.

Biosynthesis studies of RetΔ6 and Ret2A proteins have shown a slower rate of maturation for RetΔ6 protein due to precursor retention in the endoplasmic reticulum. However, both RetΔ6 and Ret2A show intracellular retention in comparison with the Retwt protein, which reaches the membrane within a 30 min chase time (data not shown). This is expected since, as a rule, only proteins that have attained a proper three-dimensional structure are secreted or expressed at the cell surface. Since RetΔ6 and Ret2A are mutated near or within Cys, their folding and assembly could potentially be delayed by intracellular retention for a longer time with respect to Retwt.

Accumulation of misfolded immature Ret2A and RetΔ6 proteins in the intracellular vesicular compartment and its homodimerization due to aberrant intermolecular disulfide bonds have important consequences on receptor functions in the downstream mitogenic program. In fact, a very large portion of RetΔ6 and Ret2A precursors strongly bind phosphorylated Shc proteins, thus indicating their ability to induce this mitogenic signal pathway. This finding may also aid in explaining why the RetΔ6 protein is more oncogenic, even when less efficiently processed than Ret2A mutant; an obvious paradox. The possibility that intracellular Ret precursors still trigger a mitogenic activity is extremely important also in the context of the effects of cysteine mutation in positions 609, 618 and 620. There is evidence suggesting that these cysteine mutations induce constitutive catalytic activity due to aberrant disulfide homodimerization of Ret, but in the same time are responsible for a decrease in the amount of mature RET protein expressed at the cell surface (Chappuis-Flament *et al.*, 1998). Since 20–30% of families with mutations C618R or C620R (Mulligan *et al.*, 1994) present a combination of HSRC and MEN 2A, it has been hypothesized that these cysteine mutations exert a dual impact on RET. In particular, these mutations can be activating or deactivating depending on the tissue in which RET is expressed. Thus, RET carrying either the C618R or C620R mutation, due to constitutive dimerization, exhibits a transforming potential responsible for MEN2A and in the same time, due to scarce localization at the surface membrane, an inability to promote a survival of enteric neurons. Our data well supports this model, indicating that not exclusively the membrane can trigger mitogenic signals. This reinforces the concept that an efficient membrane localization of Ret receptors is very important for triggering of neuronal differentiation signals.

## Materials and methods

### *Cell culture and transfection*

Mouse NIH3T3 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum. Transfection experiments were performed in NIH3T3 cells by calcium phosphate precipitation as previously described (Bongarzone *et al.*, 1993). Plasmids carrying Retwt, RetΔ6 and Ret2A inserts have been described in detail previously (Ceccherini *et al.*, 1997). The HRAS oncogene was mutated at its 12th codon by a G→T transversion. Transformation foci were selected in DMEM plus 5% calf serum. The reducing agent 2-ME was added when cells had formed a monolayer. This monolayer was maintained for 14 days in DMEM supplemented with 5% bovine calf serum.

### *Immunoprecipitation and Western blot analysis*

Protein samples were prepared as previously described (Borrello *et al.*, 1996) and immunoprecipitated with the specified antisera: affinity purified anti-Ret polyclonal antiserum (Borrello *et al.*, 1996) and rabbit polyclonal anti-Shc antiserum (Upstate Biotechnology Incorporated). Immunoprecipitates were resolved by electrophoresis on 7.5 or 8.5% SDS polyacrylamide gels (PAGE). Proteins were transferred to nitrocellulose filters, blocked with 5% bovine serum albumin (BSA) or 0.5% gelatin in Tris-buffered saline (TBS) pH 7.6 and immunoblotted with the same anti-Ret antiserum described above or with the monoclonal antiserum anti-phosphotyrosine (anti-Ptyr, Upstate Biotechnology Incorporated). Immunoreactive bands were visualized using horseradish peroxidase conjugated anti-rabbit or anti-mouse antisera and ECL detection reagents (Amersham) or using $^{125}$I-labeled protein A (Amersham) followed by autoradiography. When $^{125}$I-labeled protein A was used, filters were exposed to storage phosphor screen films (Molecular Dynamics) in order to quantify the counts per minute (c.p.m.) associated with the Ret-specific bands reacting with anti-Ret or anti-Shc with a Phosphorimager. For Western blotting under non-reducing conditions, 2-mercaptoethanol was excluded.

### *Metabolic labeling and immunoprecipitation*

Prior to labeling, subconfluent cells were incubated with methionine-free DMEM for 1 h. Metabolically labeling was performed with 370 μCi/mL $^{35}$S-Methionine-Cysteine (DuPont/NEN) for 15 min. The labeling medium was then removed and replaced with completed DMEM medium for the duration of the chase period. Further processing, extraction, and immunoprecipitation of labeled Ret was carried out as above with the following modifications: after incubation, bound immune complexes (Protein A/G-Plus-Agarose beads, Santa Cruz Biotechnology) were washed extensively (seven times) with lysis buffer (0.15 mM NaCl/0.05 mM Tris-HCl, pH 7.2/1% Triton X-100/1% sodium deoxycholate/0.1% SDS), either supplemented with or without 1% Triton and 1 M NaCl and on a shaking platform, eluted, and subjected to SDS–PAGE fractionation and autoradiography. Densities of radioactive bands on X-ray films were estimated using a Phosphorimager.

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**EXHIBIT C**

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# Ligand Stimulation of a Ret Chimeric Receptor Carrying the Activating Mutation Responsible for the Multiple Endocrine Neoplasia Type 2B*

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**Inherited activating mutations of Ret, a receptor tyrosine kinase, predispose to multiple endocrine neoplasia (MEN) types 2A and 2B and familial medullary thyroid carcinoma. To investigate the effects induced by acute stimulation of Ret, we transfected both PC12 and NIH 3T3 cells with a molecular construct in which the ligand-binding domain of the epidermal growth factor receptor was fused to the catalytic domain of Ret. Acute stimulation of the chimeric receptor induced PC12 cells to express a neuronal-like phenotype. Moreover, we introduced the dominant mutation, responsible for the multiple endocrine neoplasia type 2B, in the catalytic domain of the Ret chimera. Expression of the mutant chimera, in the absence of ligand stimulation, induces the PC12 cells to acquire a flat morphology with short neuritic processes and transforms the NIH 3T3 cells. Stimulation of the mutant chimera with epidermal growth factor causes a drastic overgrowth of long neuritic processes, with the induction of the *suc1*-associated protein tyrosine phosphorylation in PC12 cells and higher transforming efficiency in NIH 3T3 cells. These data indicate that the gain-of-function MEN2B mutation does not abrogate ligand responsiveness of Ret and suggest that the presence of Ret ligand could play a role in the pathogenesis of the MEN2B syndrome.**

Specific mutations of the *ret* gene, a receptor tyrosine kinase (1), are responsible for the inheritance of multiple endocrine neoplasia (MEN)[1] type 2A and 2B and familal medullary thyroid carcinoma syndromes (2). MEN2A and MEN2B are distinct hereditary neoplastic syndromes both characterized by the presence of medullary thyroid carcinomas and pheochromocytomas. MEN2A is also characterized by hyperplasia of parathyroid cells, whereas MEN2B is a more severe disease, being associated with skeletal abnormalities, ganglioneuromas of the intestinal tract, and mucosal neuromas, and it is characterized by an earlier age of tumor onset.(3). Mutations in cysteine residues of the extracellular domain are the causative genetic event of familal medullary thyroid carcinoma and MEN2A syndromes (4, 5). A single point mutation, which results in a Thr for Met substitution at codon 918 within the Ret catalytic domain, is responsible for the MEN2B syndrome (6, 7). These mutations convert Ret into a dominant transforming gene (*ret*MEN2A and *ret*MEN2B alleles) and cause constitutive activation of its intrinsic tyrosine kinase activity (8, 9).

*ret*MEN2A and *ret*MEN2B differ in their mechanisms of activation. In the case of *ret*MEN2A, activation likely results from constitutive receptor dimerization, whereas *ret*MEN2B proteins do not constitutively dimerize and display altered substrate specificity (2, 9, 10). It is presently unknown whether the Ret harboring the MEN2B mutation is fully activated by an intramolecular mechanism. Indeed, if *ret*MEN2B is still sensitive to ligand stimulation, the contribution of active Ret to the resulting phenotype, in the affected tissues, may be in part attributed to the presence of available Ret ligand in the extracellular environment.

We thus investigated the biological effects induced by Ret stimulation in the rat pheochromocytoma cell line, PC12, because of the sensitivity of this system, which retains the ability to differentiate *in vitro* and also allows discrimination among stimuli from different extracellular signals (11, 12). Indeed, this cell line has been particularly suitable for studying the molecular mechanisms by which *ret* alleles contribute to the development of neuroendocrine cancer syndromes (13–16). We have recently shown that chronic expression of active Ret oncoproteins induces the PC12 cells to differentiate toward a neuronal-like phenotype. Yet, we have shown that Ret-induced differentiation is not complete, because the expression of neuronal genes is dissociated from the inhibition of cell proliferation (16).

Because one of the biological mechanisms underlying the choice between differentiation and proliferation in PC12 cells is determined by the extent and duration of the signaling (12), we decided to investigate whether acute stimulation of Ret causes differentiation of the PC12 cells. In addition, we addressed the question of whether constitutive activation, induced by the MEN2B mutation, fully activates the Ret biochemical activity, thus abrogating responsiveness to ligand stimulation.

A potential physiological ligand for Ret has recently been

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[1] The abbreviations used are: MEN, multiple endocrine neoplasia; GDNF, glial cell line-derived neurotrophic factor; EGF, epidermal growth factor; EGFR, EGF receptor; NGF, nerve growth factor.

identified as the glial cell line-derived neurotrophic factor, GDNF (17–20). Ret association to GDNF and its subsequent tyrosine phosphorylation is mediated by the presence in the same cell surface complex of the GDNF receptor-α, a glycosylphosphatidylinositol-anchored protein. This protein is expressed in GDNF-responsive tissues and in cultured embryonic neurons, whereas in the cell lines examined, including PC12 and NIH 3T3 cells, complete stimulation of Ret by GDNF depends on the exogenous addition of GDNF receptor-α (19, 20). Thus, to perform experiments in PC12 cells, we utilized a chimeric receptor, EGFR/*ret*, which consists of the transmembrane and ligand-binding domains of the epidermal growth factor receptor (EGFR) fused to the catalytic domain of Ret. Such a chimeric construct has already been shown to be a useful tool in characterizing the Ret-specific transducing signaling (21, 22). In addition, we also utilized a mutant chimera, consisting of the EGFR/*ret* construct in which we introduced a single point mutation, which converts the Met codon 918 (1) into Thr (EGFR/*ret*$^{Thr-918}$ chimera).

The results reported here indicate that the MEN2B mutation does not cause full activation of Ret, since it retains the ability to be further stimulated by an extracellular ligand. This stimulation resulted in an increased autophosphorylation of the receptor, pronounced neurite outgrowth, and tyrosine phosphorylation of Snt. Thus, our results suggest that the MEN2B disease phenotype could be influenced by the tissue distribution of a Ret ligand.

## EXPERIMENTAL PROCEDURES

*Cell Culture and Transfection Experiments*—PC12 cells were grown in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% horse serum and 5% fetal calf serum (13). NIH 3T3 cells were grown in DMEM supplemented with 10% fetal calf serum (21). Transfection experiments were performed with 10 μg of plasmid DNA using either the Lipofectin reagent (Life Technologies, Inc.) for PC12 cells (13) or the calcium phosphate precipitation method for NIH 3T3 cells. The transfected cells were selected in *gpt* selection medium for 3 weeks, and individual cell colonies were isolated and expanded. Epidermal growth factor (Upstate Biotechnologies, Inc. (UBI)) or 2.5 S nerve growth factor (UBI) (100 ng/ml) were added to the culture medium as indicated.

*Immunoprecipitation and Immunoblotting*—Between $10^6$ and $10^7$ cells were washed twice in ice-cold Tris-buffered saline (20 mM Tris-HCl, pH 8.0, 150 mM NaCl) and then lysed in a buffer containing 50 mM HEPES, pH 7.5, 1% (v/v) Triton X-100, 50 mM NaCl, 5 mM EGTA, 50 mM NaF, 20 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 2 mM phenylmethylsulfonyl fluoride, 0.2 μg/ml each of aprotinin and leupeptin, and 4 mM diisopropylfluorophosphate and clarified by centrifugation at 10,000 × *g* for 15 min, as previously reported (21). Protein concentrations were estimated by a modified Bradford assay (Bio-Rad). Equal amounts of protein were incubated with rabbit anti-Ret antibody, as indicated, for 1 h at 4 °C and subsequently incubated with protein A-Sepharose CL4-B (Pharmacia) for 1 h at 4 °C. Immunoprecipitates were washed three times with the above mentioned lysis buffer and boiled in Laemmli buffer for 5 min before electrophoresis. Immunoprecipitates were subjected to SDS-PAGE (7.5% polyacrylamide) under reducing conditions and transferred to polyvinylidene difluoride (Millipore Corp.). Immunoblotting was carried out using either anti-Ret antibodies or anti-phosphotyrosine monoclonal antibodies (UBI, G410), and the reaction was detected with peroxidase-conjugated secondary antibodies and Amersham ECL system. The polyclonal antibody (anti-Ret) was generated against a fusion protein in which the tyrosine kinase domain of human Ret is fused to the bacterial glutathione *S*-transferase (21). The Snt protein was isolated from cell lysates using p13$^{suc1}$-agarose (Oncogene Science) as described (23).

*Northern Blot Analysis*—RNA was prepared from cultured cells by a modification of the guanidine thiocyanate method (24). 20 μg of total RNA were size-fractionated on a denaturing formaldehyde agarose gel and blotted onto nylon filters (Hybond-N, Amersham Corp.). To obtain the *krox24* probe, 60-mer oligonucleotides were synthesized according to the published sequence and subsequently $^{32}$P-labeled using the Klenow fragment of the *Escherichia coli* DNA polymerase and a 3′-terminal specific 9-mer. The *vgf* probe was excised from the pV2–2 plasmid (25). $^{32}$P labeling of the *vgf* and ribosomal 18 S probes was performed with the random oligonucleotide primer kit (Amersham). Hybridization and washing were carried out under stringent conditions: 0.1 × SSC, 0.1% SDS, 60 °C. Autoradiography was performed using Kodak X-AR films at −70 °C for 1–7 days with intensifying screens.

## RESULTS

*Acute Stimulation of Ret Induces Neurite Outgrowth in PC12 Cells*—PC12 cells, stably transfected with the chimera (EGFR/*ret*) or with the vector alone (LTR-3) (Fig. 1*A*), were selected for resistance to mycophenolic acid. A mass population and individual clones were then isolated and analyzed. All of the EGFR/*ret*- and vector-transfected populations appeared morphologically undifferentiated, displaying a small size and round shaped morphology (Fig. 1*B* and data not shown).

Stimulation of the EGFR/*ret* chimera with epidermal growth factor (EGF) (100 ng/ml) induced the PC12 cells to change, within 24 h, from a round shaped to a neuron-like morphology with long neurite processes that strikingly resembled that induced by the nerve growth factor (NGF) on the parental cells (Fig. 1*B*). On the other hand, EGF stimulation (up to 300 ng/ml) of the parental cells and of the vector-transfected cells had little or no effect on cell morphology even after 72 h of continuous treatment (Fig. 1*B* and data not shown).

The expression and the functional integrity of the EGFR/*ret* chimeric receptor were tested by immunoprecipitation of Ret products, followed by blotting either with anti-Ret or with anti-phosphotyrosine antibodies (Fig. 1*C*). A single protein, of 140–150-kDa apparent molecular mass, corresponding to the EGFR/*ret* product (21), was observed in the PC12-EGF/*ret* cells but not in the parental cells. In the absence of ligand stimulation, the EGFR/*ret* receptor displayed some constitutive levels of phosphorylation; however, stimulation with EGF (100 ng/ml) caused a dramatic increase (more than 20-fold) in tyrosine phosphorylation of the receptor (Fig. 1*C*).

*Ret Induces Differentiation in PC12 Cells*—NGF-induced differentiation of the PC12 cells is characterized by the expression of a complex pattern of genes, including immediate early genes (*fos*, *krox24*) or delayed and late genes (*vgf*, SCG10, peripherin), the expression of the latter genes being, at least partially, dependent on protein synthesis (26). We decided to investigate whether the Ret-induced neurite outgrowth was associated with the expression of a similar pattern of genes. In Fig. 2, we show that stimulation of the chimera induced the expression of *krox24* and *vgf* (27, 28) at levels similar to those induced by NGF. On the other hand, EGF stimulation was unable to induce any significant *vgf* expression, and it induced only low levels of *krox24* mRNA.

Because EGF is able, on its own, to induce an early gene response, which partially superimposes that induced by NGF, even if to a lower extent, we determined whether stimulation of the chimera could induce tyrosine phosphorylation of a specific target of neurotrophic factor activity in the neuronal cells, Snt (Fig. 3). Tyrosine phosphorylation of Snt has been reported as a qualitative event that discriminates between proliferation signals, induced by serum or EGF, and differentiation signals, such as that induced by NGF, even if its biological function is still poorly understood (23, 29). EGF stimulation of the EGFR/*ret* chimera resulted in tyrosine phosphorylation of Snt at levels similar to those observed following stimulation with NGF (Fig. 3, compare *lane 5* to *lanes 3* and *6*). On the other hand, EGF had no effect on the parental PC12 cells (Fig. 3, *lane 2*).

*Ligand Stimulation Increases the retMEN2B Activity in PC12 and NIH 3T3 Cells*—A single point mutation in the catalytic domain of Ret, which is associated with the MEN2B syndrome, causes constitutive tyrosine kinase activation. This mutation enables Ret to transform the NIH 3T3 cell line and to cause incomplete differentiation of the PC12 cells (9, 16). To

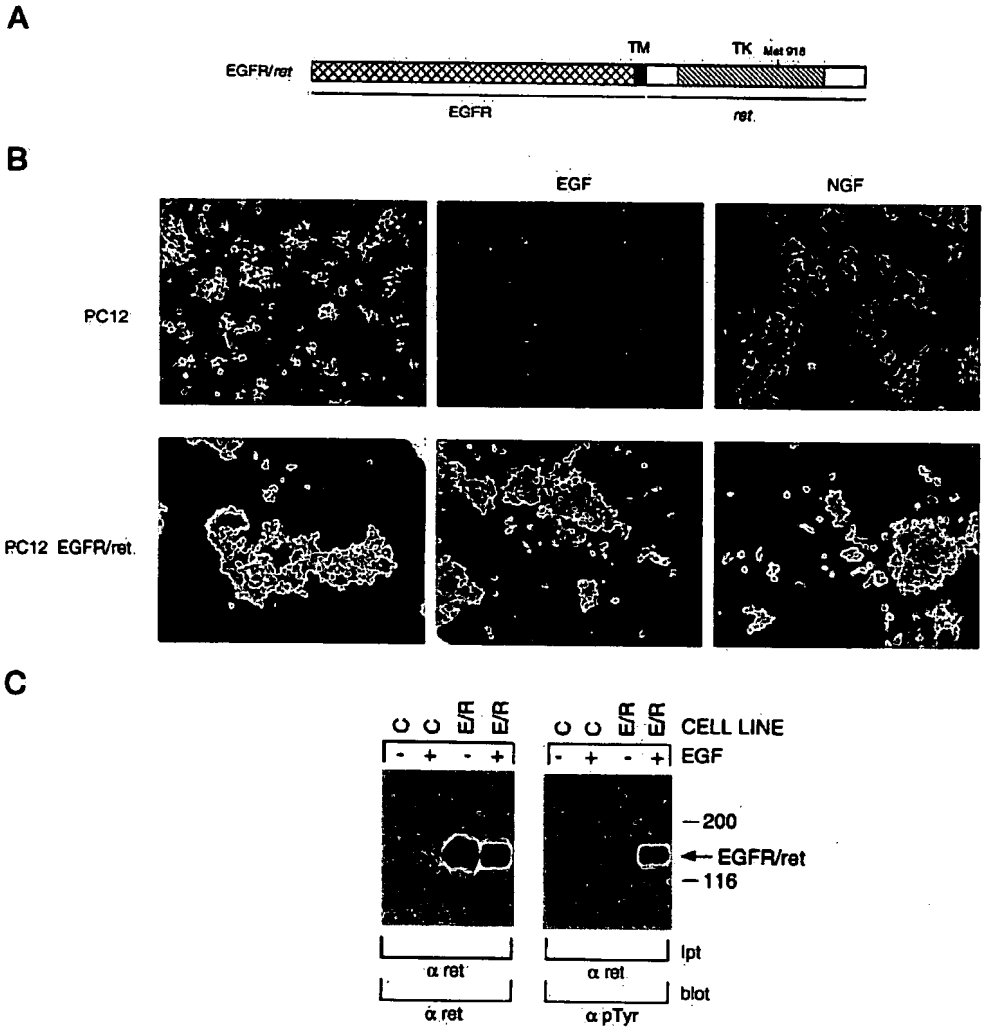


FIG. 1. *Panel A*, schematic representation of the EGFR/*ret* construct. The EGFR/*ret* chimera encompasses the extracellular and transmembrane domains of the EGFR, and the intracellular domain of Ret (21). Met-918 is also indicated. *Panels B* and *C*, effects of ligand-dependent stimulation of the EGFR/*ret* chimera on PC12 cell morphology and phosphorylation of the receptor. *Panel B*, phase contrast micrograph of parental PC12 cells (*upper parts*) or EGFR/*ret* (*lower parts*) transfectants. Cells were grown for 72 h in the presence of either NGF (100 ng/ml) or EGF (100 ng/ml) or left untreated as indicated. EGF induces neurite outgrowth in EGFR/*ret* transfectants, which are similar to those observed in the parental cells treated with NGF. *Panel C*, total cellular proteins were immunoprecipitated (*Ipt*) with the anti-Ret polyclonal antibody (*αret*) and then divided into two aliquots and analyzed by immunoblotting (*blot*) with the anti-Ret or anti-Tyr(P) (*αpTyr*) antibody, as indicated. Control mock-transfected PC12 cells (*C*) or EGFR/*ret* transfectant (*E/R*) were either untreated (−) or treated with 100 ng/ml EGF for 5 min at 37 °C (+). Molecular mass markers are indicated in kilodaltons. The position of the EGFR/*ret* chimera is also indicated.

address the question of whether the *ret*MEN2B is further inducible, we introduced the Met-918 to Thr substitution in the EGFR/*ret* construct (thus obtaining the EGFR/*ret*$^{Thr-918}$). We first evaluated its effects in NIH 3T3 cells in a focus formation assay. Consistent with the "gain of function" effects of the MEN2B mutation (9), EGFR/*ret*$^{Thr-918}$ transformed also in the absence of EGF ($10^2$ focus-forming units/pmol). EGF stimulation further increased the transforming activity of the EGFR/*ret*$^{Thr-918}$ construct (Table I), indicating that also in the presence of a MEN2B mutation, Ret retained responsiveness to ligand triggering.

PC12 cells were then transfected with the EGFR/*ret*$^{Thr-918}$ construct, and both a mass population and several independent clones were marker-selected. The morphology of PC12-EGFR/*ret*$^{Thr-918}$ cells was indistinguishable from that previously reported in the case of PC12 cells expressing a *ret*MEN2B allele (16). Indeed, PC12-EGFR/*ret*$^{Thr-918}$ cells were flat and showed the growth of short neurites (Fig. 4). However, they were still responsive to ligand triggering. Twenty-four hours of EGF treatment induced the PC12-EGFR/*ret*$^{Thr-918}$ cells to shift toward a more differentiated neuronal phenotype that was, however, clearly different from that induced by NGF on parental cells. As shown in Fig. 4, although EGF induced a pronounced neuritic outgrowth, PC12-EGFR/*ret*$^{Thr-918}$ cells still retained a flat shaped cell body that contrasted with the round shape and the high refractility characterizing PC12 cells treated with NGF.

These biological effects were explained by the retained responsiveness of the tyrosine kinase activity of the EGFR/*ret*$^{Thr-918}$ construct to ligand stimulation. Consistent with the reported constitutive activation of the tyrosine kinase function of Ret caused by the MEN2B mutation (9), the EGFR/*ret*$^{Thr-918}$ protein product showed constitutive levels of tyrosine phosphorylation, both in NIH 3T3 and PC12 cells, which were higher than those of the wild type EGFR/*ret* chimera (Fig. 5*A* and data not shown). However, EGF stimulation caused a sharp increase in the phosphorylation of the receptor in both cell lines. On the other hand, phosphorylation of Snt seems to correlate with the levels of tyrosine phsphorylation of the receptor. In fact, Snt was barely phosphorylated in cells transfected with EGFR/

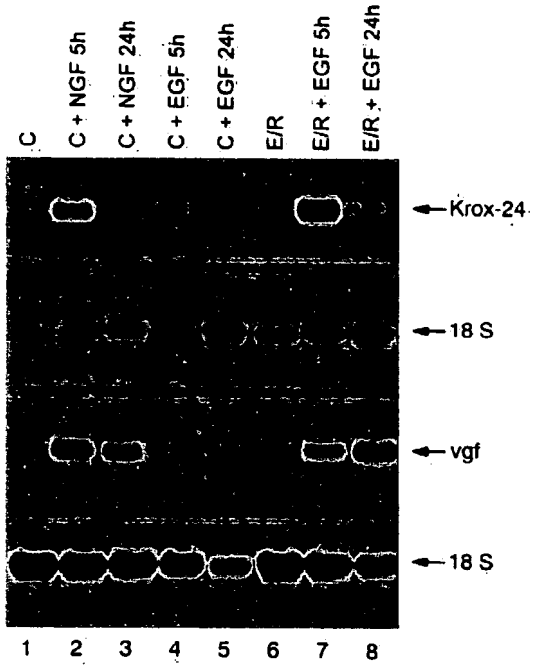


FIG. 2. **Gene expression induced by EGFR/*ret* in PC12 cells.** Northern blot analysis is shown of total cellular RNA (20 μg) extracted either from PC12 cells (*lanes 1–5*) or from PC12 cells transfected with EGFR/*ret* (*lanes 6–8*), grown in the presence of NGF (100 ng/ml) or EGF (100 ng/ml) as indicated. The filters were hybridized with either a *krox24*-specific, or a *vgf*-specific probe as indicated. Equal gel loading was confirmed by the hybridization with an 18 S-specific ribosomal RNA probe. These results are representative of three independent experiments.

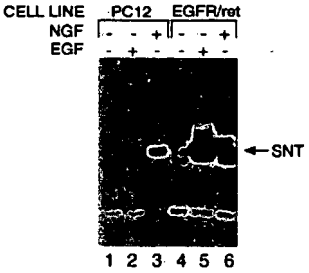


FIG. 3. **EGFR/*ret* stimulation of tyrosine phosphorylation of Snt in PC12.** Cells were untreated (*lanes 1* and *4*) or treated with 100 ng/ml NGF (*lanes 3* and *6*) or EGF (*lanes 2* and *5*) for 5 min at 37 °C. Cell lysates were incubated with p13$^{suc1}$-agarose, eluted, and analyzed by immunoblot with anti-Tyr(P) (*apTyr*) antibody. The position of Snt is indicated.

*ret*$^{Thr-918}$, whereas stimulation of the mutant chimera caused its marked tyrosine phosphorylation (Fig. 5*B*) and overinduction of *krox24* gene expression (not shown).

## DISCUSSION

Here we report data showing that the *ret* gene is able to differentiate the PC12 cells and that Ret carrying the MEN2B activating mutation is further inducible by ligand stimulation. To perform this study, we took advantage of an inducible system represented by a chimeric receptor (EGFR/*ret*) in which the tyrosine kinase activity of Ret was triggerable by EGF. When stimulated with EGF, PC12 cells transfected with EGFR/*ret* acquired a neuronal phenotype, characterized by long neuritic processes and the expression of immediate (*krox24*) as well as delayed (*vgf*) genes. Such phenotype is undistinguishable from that induced by NGF ("NGF phenotype"). This was further supported by the observation that the EGFR/*ret* chimera was also able to induce the tyrosine phosphorylation of Snt, a molecule that is regarded as a specific target of neurotrophic factors (23, 29).

TABLE I
*Transforming activity of the EGFR/ret$^{Thr-918}$ chimera in NIH 3T3 cell fibroblasts*

| Transfected DNA | Transformation of NIH 3T3 cells[a] | |
|---|---|---|
| | −EGF | +EGF |
| | *FFU/pmol DNA* | |
| EGFR/*ret* | $1 \times 10^1$ | $1 \times 10^3$ |
| EGFR/*ret*$^{Thr-918}$ | $1 \times 10^2$ | $4 \times 10^3$ |
| LTR-3 | $<1 \times 10^1$ | $<1 \times 10^1$ |

[a] Transfections were performed using 40 μg of carrier calf thymus DNA. Where indicated, EGF (20 ng/ml) was added after 14 days. Focus-forming activity (in focus-forming units (FFU)) was scored at day 21 on duplicate plates transfected with 10-fold dilutions of the DNA of interest. Transforming activity is corrected for the efficiency of transfection calculated in parallel plates subjected to marker selection. Results are the means of three experiments performed in duplicate.

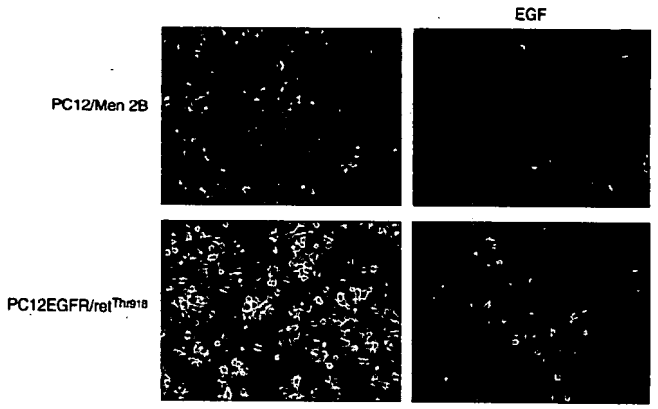


FIG. 4. **Effects of stimulation of the EGFR/*ret*$^{Thr-918}$ chimera on PC12 cell morphology.** A phase contrast micrograph of *ret*$^{Thr-918}$ (*upper panels*) or EGFR/*ret*$^{Thr-918}$ (*lower panels*) PC12 cell transfectants is shown. A marker-selected mass population was grown for 72 h either in the absence or presence of EGF (100 ng/ml) as indicated. The same results were observed when four independent cell clones were analyzed for each cell line.

The pattern of neuronal gene induction in PC12 cells, expressing the chronically active *ret*MEN2A and *ret*MEN2B alleles, is similar to that elicited by the acute stimulation of the EGFR/*ret* chimera. However, PC12-*ret*MEN2A and PC12-*ret*MEN2B cells displayed a less differentiated morphology with respect to EGF-stimulated PC12-EGFR/*ret* cells, since the former were characterized by a flat cell body and short neuritic processes ("MEN2 phenotype") (16) and the latter displayed a NGF phenotype. Whether or not these differences resulted from the kinetics of activation of the forms used, namely acute stimulation of EGFR/*ret versus* chronic activation of *ret*MEN2A and *ret*MEN2B, remains to be determined.

The inheritance of specific *ret* mutations causes distinct disease phenotypes, thus suggesting that some specific cell types undergo abnormal proliferation depending on the type of *ret* activation (*via* a MEN2A or *via* a MENB mutation) (2, 3). One possibility is that *ret*MEN2B activity could still be influenced by cell- or tissue-specific biological constraints, such as, for example, the density of the available ligand.

We thus investigated this possibility by using a mutated version of the chimera (EGFR/*ret*$^{Thr-918}$), harboring the MEN2B mutation. Consistent with the notion that MEN2B causes a gain of function of Ret, the EGFR/*ret*$^{Thr-918}$ construct was able to transform NIH 3T3 cells and induce differentiation in PC12 cells, even in the absence of EGF. PC12 cells transfected with EGFR/*ret*$^{Thr-918}$ showed a phenotype indistinguishable from PC12 transfected with *ret*MEN2B, thus confirming

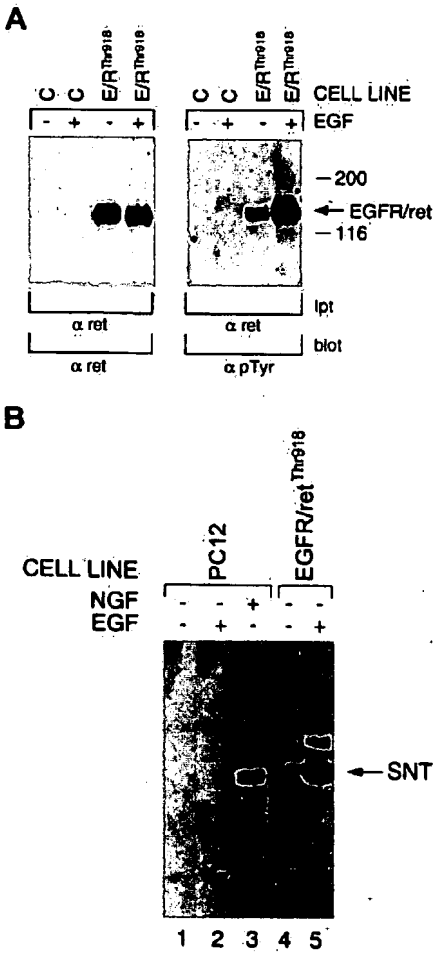


FIG. 5. **Stimulation of the EGFR/*ret*$^{Thr\text{-}918}$ induces tyrosine phosphorylation of the receptor and mediates tyrosine phosphorylation of Snt in PC12 cells.** *Panel A*, total cellular proteins from PC12 transfectants were immunoprecipitated (*Ipt*) with the anti-Ret polyclonal antibody (*αret*) and analyzed by immunoblotting (*blot*) with the anti-Ret or anti-Tyr(P) (*αpTyr*) antibody, as indicated. Control untransfected cells (*C*), EGFR/*ret* (*E/R*), and EGFR/*ret*$^{Thr\text{-}918}$ (*E/R*$^{Thr\text{-}918}$) transfectants were either untreated (−) or treated with 100 ng/ml EGF (+) for 5 min at 37 °C. Molecular mass markers are indicated in kilodaltons. The position of the EGFR/*ret* chimera is also indicated. *Panel B*, PC12 or PC12 cell transfectants were untreated (*lanes 1*, *4*, and *6*) or treated with 100 ng/ml EGF (*lanes 2* and *5*), or 100 ng/ml NGF (*lane 3*) for 5 min at 37 °C. Cell lysates were incubated with p13$^{suc1}$-agarose, eluted, and analyzed by immunoblot with anti-Tyr(P) (*αpTyr*) antibody. The position of Snt is indicated.

that the expression in PC12 cells of a constitutive active Ret version results in a MEN2 phenotype. It is noteworthy that the MEN2B mutation was less effective in activating Ret function when cloned in the EGFR/*ret* construct with respect to the full-length *ret*. Since the difference between EGFR/*ret* and *ret* resides in their extracellular and transmembrane domains, it is likely that some specific characteristics of such domains confer to Ret this particular susceptibility to the activating effect of the MEN2B mutation.

However, despite the fact that the Met-918 to Thr mutation constitutively activates the chimera, the biological effects of the EGFR/*ret*$^{Thr\text{-}918}$ construct were markedly sensitive to EGF triggering. EGF stimulation caused a marked increase of the transforming ability of the EGFR/*ret*$^{Thr\text{-}918}$ construct and modified the phenotype of PC12-EGFR/*ret*$^{Thr\text{-}918}$ cells, determining the overgrowth of long neuritic processes and a dramatic phosphorylation of Snt. These effects were consistent with the stimulation of tyrosine phosphorylation of EGFR/*ret*$^{Thr\text{-}918}$ caused by EGF.

These results show that the MEN2B mutation does not abrogate ligand responsiveness of Ret. However, we cannot discriminate whether the stimulation of the mutated Ret enhances the activity of the receptor, without changing the substrate specificity or, more likely, uncovers docking sites for new substrates. This ligand responsiveness may have important implications in the human diseases associated with *ret*MEN2B mutations. It is likely that some of the differences in the disease phenotype between MEN2A and MEN2B syndromes could depend on the tissue distribution of the Ret ligand and on the different susceptibility of *ret*MEN2A and *ret*MEN2B alleles to the action of such a ligand.

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**EXHIBIT D**

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**Resources**

**1:** Ret — Links

Ret oncogene [*Drosophila melanogaster*]
**Other Aliases:** Dmel_CG14396, CG1061, CG14396, D-ret, DRET, DmHD-59, MEN2, RET
**Other Designations:** Ret oncogene CG14396-PA, isoform A; Ret oncogene CG14396-PB, isoform B; Ret oncogene CG14396-PC, isoform C; Ret oncogene CG14396-PD, isoform D; Ret oncogene CG14396-PE, isoform E
**Chromosome:** 2L; **Location:** 39B1-39B1
**GeneID:** 43875

**2:** RET — Order cDNA clone, Links

**Official Symbol** RET and **Name:** ret proto-oncogene [*Homo sapiens*]
**Other Aliases:** CDHF12, HSCR1, MEN2A, MEN2B, MTC1, PTC, RET-ELE1, RET51
**Other Designations:** Hirschsprung disease 1; RET transforming sequence; cadherin family member 12; hydroxyaryl-protein kinase; multiple endocrine neoplasia and medullary thyroid carcinoma 1; oncogene RET; receptor tyrosine kinase; ret proto-oncogene (multiple endocrine neoplasia and medullary thyroid carcinoma 1, Hirschsprung disease)
**Chromosome:** 10; **Location:** 10q11.2
**Annotation:** Chromosome 10, NC_000010.9 (42892533..42944955)
**MIM:** 164761
**GeneID:** 5979

**3:** Ret — Links

**Official Symbol** Ret and **Name:** ret proto-oncogene [*Rattus norvegicus*]
**Other Designations:** Ret gene for receptor tyrosin; Ret proto-oncogene (multiple endocrine neoplasia MEN2A MEN2B and medullary thyroid carcinoma 1 Hirschsprung disease); receptor tyrosine kinase
**Chromosome:** 4; **Location:** 4q42
**Annotation:** Chromosome 4, NC_005103.2 (154448179..154491103, complement)
**GeneID:** 24716

**4:** RET — Links

ret proto-oncogene [*Canis lupus familiaris*]
**Other Designations:** ret proto-oncogene (multiple endocrine neoplasia and medullary thyroid carcinoma 1, Hirschsprung disease); ret tyrosine kinase
**Chromosome:** 28

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**Annotation:** Chromosome 28, NC_006610.2 (6945076..6976159, complement)
**GeneID:** 403494

**5:** Ret Order cDNA clone, Links

**Official Symbol** Ret and **Name:** ret proto-oncogene [*Mus musculus*]
**Other Aliases:** PTC, RET51, RET9, c-Ret
**Chromosome:** 6; **Location:** 6 53.2 cM
**Annotation:** Chromosome 6, NC_000072.5 (118101766..118147762, complement)
**GeneID:** 19713

**6:** RET Links

ret proto-oncogene [*Gallus gallus*]
**Chromosome:** 6
**Annotation:** Chromosome 6, NC_006093.2 (5877654..5953679, complement)
**GeneID:** 396107

**7:** RET Links

ret proto-oncogene [*Pan troglodytes*]
**GeneID:** 449594

**8:** LOC450415 Links

similar to ret proto-oncogene isoform c; hydroxyaryl-protein kinase; RET transforming sequence; oncogene RET; cadherin family member 12 [*Pan troglodytes*]
**Chromosome:** 10
**GeneID:** 450415
This record was discontinued.

**9:** ret

reticulated [*Drosophila melanogaster*]
**Other Aliases:** FBgn0010168
**Chromosome:** 1; **Location:** 1-
**GeneID:** 44319
This record was discontinued.

**10:** RFPL4A Links

**Official Symbol** RFPL4A and **Name:** ret finger protein-like 4A [*Homo sapiens*]

**EXHIBIT E**

## 1: RET ret proto-oncogene [ *Homo sapiens* ]

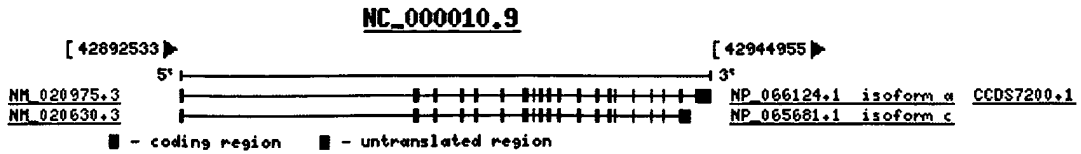
GeneID: 5979 updated 16-Sep-2007

### Summary

| | |
|---|---|
| **Official Symbol** | RET provided by HGNC |
| **Official Full Name** | ret proto-oncogene provided by HGNC |
| **Primary source** | HGNC:9967 |
| **See related** | Ensembl:ENSG00000165731; HPRD:01266; MIM:164761 |
| **Gene type** | protein coding |
| **RefSeq status** | Reviewed |
| **Organism** | *Homo sapiens* |
| **Lineage** | *Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi; Mammalia; Eutheria; Euarchontoglires; Primates; Haplorrhini; Catarrhini; Hominidae; Homo* |
| **Also known as** | PTC; MTC1; HSCR1; MEN2A; MEN2B; RET51; CDHF12; RET-ELE1 |
| **Summary** | This gene, a member of the cadherin superfamily, encodes one of the receptor tyrosine kinases, which are cell-surface molecules that transduce signals for cell growth and differentiation. This gene plays a crucial role in neural crest development, and it can undergo oncogenic activation in vivo and in vitro by cytogenetic rearrangement. Mutations in this gene are associated with the disorders multiple endocrine neoplasia, type IIA, multiple endocrine neoplasia, type IIB, Hirschsprung disease, and medullary thyroid carcinoma. Two transcript variants encoding different isoforms have been found for this gene. Additional transcript variants have been described but their biological validity has not been confirmed. |

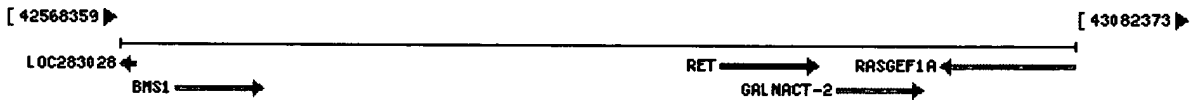
### Genomic regions, transcripts, and products

Go to reference sequence details



## Genomic context

**chromosome**: 10; **Location:** 10q11.2 See RET in MapViewer




## Bibliography

### Related Articles in PubMed

PubMed links

### GeneRIFs: Gene References Into Function

1. These data highlight the pivotal role of the RET gene in both isolated and syndromic Hirschsprung disease.
2. Of these 86 variations, 8 proved to be in regions highly conserved among different vertebrates and within putative transcription factor binding sites. We therefore considered these as candidate disease-associated variants.
3. frequency of RET rearrangements in papillary thyroid carcinoma for the Polish population
4. our results suggest that SPRY2 regulates GDNF-dependent proliferation and differentiation of TGW neuroblastoma cells mediated by RET tyrosine kinase.
5. RET/PTC rearrangement in thyroid tumors. Review
6. Point mutation in exon 14 at codon 804 of the RET proto-oncogene locus in a case of lymph node metastases of medullary and papillary thyroid carcinoma.
7. High prevalence of BRAF mutations in thyroid cancer is genetic evidence for constitutive activation of th RET/PTC-RAS-BRAF signaling pathway in papillary thyroid carcinoma.
8. The Cys630 RET genotype may have a more vigorous transforming activity than currently thought and

can cause medullary thyroid carcinoma in RET gene carriers within the first year of life.

9. RET, a receptor tyrosine kinase involved with differentiation, was consistently up-regulated throughout the time course of retinoic acid treatment in the majority of neuroblastic tumor cell lines.
10. Findings identify RET as a novel substrate of PTPRJ and suggest that PTPRJ expression levels may affect tumor phenotype associated with RET/PTC1 and RET(C634R) mutants.
11. Copy gain of PDGFB occurs in a subset of tumors showing no evidence of mutated BRAF or rearranged ret, suggesting that copy gain of PDGFB may underlie the increased expression of platelet-derived growth factor described recently in the literature.
12. Mutations of RET proto-oncogene may play an important role in the pathogenesis of Chinese patients with Hirschsprung disease.
13. RET gene mutation may explain the wide clinical variability associated with germline mutations at codor 804 in medullary thyroid carcinoma/multiple endocrine neoplasia type 2A patients.
14. The RET/PTC1 oncogene activates a proinflammatory program, provide a direct link between a transforming human oncogene, inflammation, and malignant behavior.
15. Mutagenesis analysis revealed that Tyr981 within the intracellular domain of RET was crucial for the interaction with SH2-Bbeta. Morphological evidence showed that SH2-Bbeta and RET colocalized in mesencephalic neurons.
16. Germ-Line Mutation in RET proto-oncogene is associated with Multiple Endocrine Neoplasia
17. Three new somatic cell missense mutations of the RET proto-oncogene associated with sporadic medullary thyroid carcinoma (MTC).
18. C630R mirrors C634R in penetrance and in early age of onset of medullary thyroid carcinoma
19. RET rearrangements may not play any distinctive role in driving histotype development and cancer progression in papillary thyroid carcinomas.
20. in RET mutation carriers in Hirschsprung's disease, the gut caliber change was almost identical to the histologic transition in cases of short segment aganglionosis, whereas these were markedly dissociated in cases exhibiting extensive aganglionosis
21. in the presence of RET oncoproteins, both RAI and GAB 1 are tyrosine-phosphorylated, and the stoichiometry of this interaction remarkably increases

22. Analysis of the RET gene revealed neither linkage nor mutations in Hirschsprung's disease mapping.
23. new missense point mutation in exon 8 of the RET gene (1597G-->T) corresponding to a Gly(533)Cys substitution in the cysteine-rich domain of RET protein
24. Not only RET mutations but also RET polymorphic variants may contribute to the occurrence of total intestinal aganglionosis.
25. Single nucleotide polymorphisms in the RET oncogene may play a role in sporadic papillary thyroid carcinoma.
26. A founding locus within the RET proto-oncogene may account for a large proportion of apparently sporadic Hirschsprung disease and a subset of cases of sporadic medullary thyroid carcinoma
27. Identification of a heterozygous germ line missense mutation at codon 634 of exon 11 in the RET gene that causes a cysteine to arginine amino acid substitution in a MEN2A patient.
28. Dissecting Hirschsprung disease. RET is the main gene conferring susceptibility.
29. Segregation at three loci explains familial and population risk in Hirschsprung disease. We show oligogenic inheritance of S-HSCR, the 3p21 and 19q12 loci as RET-dependent modifiers, and a parent-of-origin effect at RET.
30. Patients with RET codon 790/791 mutations seemed to have a less aggressive clinical course compared with patients with classic multiple endocrine neoplasia type 2A syndrome.
31. germline mutation of the RET proto-oncogene in members of Slovak families with multiple endocrine neoplasia 2
32. Single nucleotide polymorphism in ret is associated with the aggressive growth of pancreatic cancers
33. Amplification and overexpression of mutant RET in multiple endocrine neoplasia type 2-associated medullary thyroid carcinoma. RET germline mutation in codon 634. Tandem duplication.Genomic chromosome 10 abnormalities increase mutant RET mRNA.
34. Expression of the mitogenic and invasive phenotype of RET/PTC-transformed thyroid cells is stimulated by osteopontin.
35. Data report the crystal structure of GFRalpha1 domain 3, and the effects of specific mutations on GDNF binding and RET phosphorylation.

36. RET/PTC expression phosphorylates the Y701 residue of STAT1, a type II interferon (IFN)-responsive protein.
37. the absence of RET alterations in all cases of C-cell hyperplasia
38. Loss-of-function germline mutations of the RET proto-oncogene are reported in familial and sporadic cases of Hirschsprung disease (HSCR) with a variable frequency
39. Selective disruption of oncogenic RET signaling in medullary thyroid carcinoma in vitro and in vivo is associated with loss of the neoplastic phenotype of medullary thyroid carcinoma.
40. RET expression leads to increased HSF1 activation, which correlates with increased expression of stress response genes. RET may be directly responsible for expression of stress response proteins and the initiation of stress response.
41. RET has roles in neoplastic transformation [review]
42. Papillary carcinomas with high RET/PTC1 expression showed an association trend for large tumor size.
43. direct interaction between RET and a broad range of effector molecules that may contribute to tumor pathogenesis
44. gene expression impairment seems to be at the basis of the association of HSCR disease with several RET polymorphisms, allowing us to define a predisposing haplotype spanning from the promoter to exo 2.
45. mutations of the RET protooncogene were analyzed in Russian patients with inherited or sporadic medullary thyroid carcinoma.The most common mutation affected codon 918 to cause substitution of methionine with threonine and accounted for 31.6% alleles.
46. The finding of a somatic deletion in RET exon 15 clarified the sporadic nature of a medullary thyroid carcinoma suspected to be familial. A 12 bp deletion within the catalytic domain of the protooncogene RET.
47. Possible pathogenesis of papillary thyroid carcinoma caused by exon 13 and 14 RET mutations that affect the intracellular domain of ret proto-oncogene protein.
48. Hirschsprung associated GDNF mutations do not prevent RET activation
49. Koreans showed increased RET gene expression in papillary thyroid carcinoma.

50. Nuclease-resistant aptamers that recognize the human receptor tyrosine kinase RET were obtained usin RET-expressing cells as targets in a modified random pool of sequences.
51. RET point mutants for follicular thyroid cells may account for the occurrence of papillary thyroid carcinoma in patients affected by familial medullary thyroid carcinoma
52. RET signals through focal adhesion kinase in medullary thyroid cancer cells.
53. RET/PTC is able to phosphorylate the Y315 residue of PKB, an event that results in maximal activation c PKB for RET/PTC-induced thyroid tumorigenesis.
54. possibility that lower-penetrance RET mutations may contribute to the list of causes of familial pheochromocytomas
55. Germline mutation on the RET gene was present in patients with pheochromocytoma or functional paraganglioma.
56. Two Hirschsprung disease-associated haplotypes derive from a single founding locus, extending up to intron 19 and successively rearranged in correspondence with a high recombination rate region located between the proximal and distal end of the RET gene.
57. These results reveal novel roles of key RET-dependent signaling pathways in embryonic kidney development and provide murine models and new insights into the molecular basis for CAKUT.
58. Mutational screening of RET revealed 9 different mutations, present in 26 of the 114 MEN 2 Spanish patients.
59. Timing and extent of prophylactic thyroidectomy can be modified by individual RET mutation
60. RET dysfunction has a crucial role and discusses RET as a potential therapeutic target.
61. These results suggest that RFP is a mediator connecting several MBD proteins and allowing the formation of a more potent transcriptional repressor complex.
62. Children of families with RET cysteine mutations may develop early metastatic medullary carcinoma of the thyroid gland.
63. study demonstrates that the interaction between RET and PHOX2B polymorphisms has a substantial impact on risk of Hirschsprung's disease
64. The BRAF(V599E) mutation appears to be an alternative event to RET/PTC rearrangement rather than t

RAS mutations, which are rare in PTC. BRAF(V599E) may represent an alternative pathway to oncogeni MAPK activation in PTCs without RET/PTC activation.

65. Letter discussing RET mutations in distinguishing between sporadic and familial medullary thyroid carcinoma.

66. the RET proto-oncogene mutation Y791F, characterized by a low penetrance, occurs comparatively frequently among patients with normal serum calcitonin concentrations

67. if the association between Hashimoto's thyroiditis and thyroid cancer exists, its molecular basis is different from RET/PTC rearrangement

68. Specific nucleotide and amino acid exchanges at codon 634 might have a direct impact on tumor aggressiveness in MEN 2A.

69. All Ret dominant-negative/+ mice died by 1 month of age and had distal intestinal aganglionosis reminiscent of Hirschsprung disease (HSCR) in humans

70. Mutations in medullary thyroid cancer and in multiple endocrine neoplasia 2.

71. The newly identified RET/N777S germline mutation is a low-penetrant cause of medullary thyroid carcinoma.

72. Medullary thyroid carcinoma as part of multiple endocrine neoplasia type 2 in a family with a mutation i RET proto oncogene.

73. results indicate a possible association between the presence of lymph node involvement at the time of diagnosis (extent of disease) of medullary thyroid carcinoma and L769L or S836S polymorphism.

74. Differentiation of cardiac ganglionic cells is affected, after RETINOIC ACID treatment, by the down-regulation of c-Ret.

75. RET requires coupling of Gab1 to phosphatidylinositol 3-kinase for function in human tumor cells

76. Expression of a human Ret proto-oncogene with the MEN 2B mutation does not cause any features of MEN 2B in mice.

77. RET/PTC and CK19 have roles in progression of papillary thyroid carcinoma

78. Data show that the the RET receptor (RET/PTC), Ras and BRAF function along a linear oncogenic signaling cascade in which RET/PTC induces RAS-dependent BRAF activation and RAS- and BRAF-

dependent ERK activation.

79. early detection of RET proto-oncogene mutation is crucial for prevention of thyroidectomy in multiple endocrine neoplasia type 2 children

80. Y1062 is a critical regulator of Ret9 signaling and suggest that Ret51-specific motifs are likely to inhibit the activity of this isoform

81. new missense point mutation in exon 5 in familial medullary thyroid carcinoma

82. Ret tyrosine 981 constitutes the major binding site of the Src homology 2 domain of Src and therefore the primary residue responsible for Src activation upon Ret engagement

83. With this study we excluded influence of the G691S polymorphism on RET mRNA expression, development of somatic RET mutation, the linkage with germline RET mutation, younger onset of medullary thyroid carcinoma, and clinical outcome of the disease.

84. RET activation closely parallels the morphological changes, that it is restricted to those areas of the tumor with the cytological alterations and that it is detectable in both mono- and polyclonal tumors

85. Shp2 activity required for RetM918T-induced Akt activation. Shp2 downstream mediator of mutated receptors RetC634Y and RetM918T. Shp2 acts as limiting factor in Ret-associated endocrine tumors, in neoplastic syndromes multiple endocrine neoplasia.

86. Persephin/GFRalpha4 is unable to recruit RET protein into lipid rafts.

87. Single nucleotide polymorphisms of RET is associated with Hirschsprung disease

88. A novel Val648Ile substitution in RET protooncogene observed in a Cys634Arg multiple endocrine neoplasia type 2A kindred presenting with an adrenocorticotropin-producing pheochromocytoma.

89. Substantial discrimination between predicted functional classes of RET mutations and disease severity even for a multigenic disease such as Hirschsprung disease.

90. The histone acetylation level was evaluated by the chromatin immunoprecipitation method applied to cells displaying different degrees of endogenous RET expression.

91. evaluation of noncoding sequences at the zebrafish ret locus conserved among teleosts, and at the human RET locus, conserved among mammals

92. Cell type-specific functions involve a competitive recruitment of different phosphotyrosine binding

adaptor molecules by RET that activate selective signaling pathways.

93. Ret mutations in thyroid tumorigenesis.
94. tumor samples from FMTC patients showed strong nuclear staining of phosphorylated ERK1/2 and Ser (727) STAT3; FMTC-RET mutants activate a Ras/ERK1/2/STAT3 Ser(727) pathway, which plays an important role in cell mitogenicity and transformation.
95. A germline RET mutation at codon 603 in exon 10 is associated with both medullary and nonmedullary thyroid cancer in a kindred.
96. there is a low-penetrance pheochromocytoma susceptibility locus in a region upstream of the putative loci for Hirschprung disease and apparently sporadic thyroid carcinoma.
97. RET oligonucleotide microarray for the detection of RET mutations in multiple endocrine neoplasia type syndromes
98. 5'-End RET splicing: absence of variants in normal tissues and intron retention in pheochromocytomas.
99. Dok-6 binds to the phosphorylated Ret Tyr(1062) residue resulting in phosphorylation of tyrosine residue(s) located within the unique C terminus of Dok-6 predominantly through a Src-dependent mechanism
100. relationship between RET oncogene and Chinese patients with Hirschsprung's disease
101. mutated in papillary thyroid cancer.
102. These observations lend support to the idea that both RET alleles have a role in pathogenesis of Hirschsprung's disease, in a dose-dependent fashion. We also showed that the c135G/A polymorphism modifies the phenotype.
103. determination of mutation specific gene expression profiles in papillary thyroid carcinoma
104. Erk8 has a role as a novel effector of RET/PTC3 and, therefore, RET biological functions
105. First molecular studies on a complex germline RET mutation lying in the juxtamembrane region of the receptor are reported in medullary thyroid carcinoma
106. The molecular basis for HPT has been further elucidated by teh detection of inactivating germline mutations in the CaSR gene in familial hypocalciuric hypercalcemia syndrome and in the RET genes in the familial forms of HPT.

107. These findings provide evidence for a novel cooperative interaction between VEGFR2 and RET that mediates VEGF-A functions in ureteric bud cells.
108. RET codon 691 polymorphism is associated with radiation induced tumors with a C-cell hyperplasia of thyroid tumors
109. role in regulating rac activity and lamellipodia formation
110. genetic interaction between mutations in RET and EDNRB is an underlying mechanism for Hirschsprung disease
111. RET expression in papillary thyroid cancer from patients irradiated in childhood for benign conditions.
112. family in which the MEN 2A and the HSCR phenotypes are associated with a single point mutation in exon 10 of the RET proto-oncogene. polymorphic sequence variants of the RET proto-oncogene.
113. a protective role of this low-penetrant haplotype in the pathogenesis of HSCR and demonstrate a possible functional effect linked to RET messenger RNA expression.
114. analysis of RET somatic mutations supports the differentiation between sporadic and inherited medullar thyroid carcinoma
115. Analysis of mutation of protooncogene RET are presented in patients with thyroid medullary carcinoma
116. Four novel intronic mutations that have a strong association with the HSCR phenotype were identified ir Hirschsprung disease patients
117. the G691S and S904S variants of RET may somehow play a role on the age of onset of MEN 2A
118. PHOX2A, but not PHOX2B, seems to act directly on the c-RET promoter
119. RET/PTC associates with STAT3 and activates it by the specific phosphorylation of the tyrosine 705 residue. STAT3 activation by the RET/PTC tyrosine kinase is one of the critical signaling pathways for th regulation of specific genes.
120. medullary thyroid carcinoma manifested in new RET mutation and RET polymorphism.
121. Familial medullary thyroid carcinoma: clinical variability and low aggressiveness associated with RET mutation at codon 804.
122. Activation of RET tyrosine kinase regulates interleukin-8 production by multiple signaling pathways

123. A deletion of the chromosomal region including the RET proto-oncogene is involved in the pathogenesis of SCLC
124. Mass spectrometric analysis revealed that RET Tyr(806), Tyr(809), Tyr(900), Tyr(905), Tyr(981), Tyr (1062), Tyr(1090), and Tyr(1096) were autophosphorylation sites.
125. RET enhancer modulates expression in the enteric nervous system consistent with its proposed role in Hirschsprung disease
126. significant association of the S691 allele with medullary thyroid carcinoma
127. Characterization of the R833C substitution suggests that this tyrosine kinase mutation confers a weak activating potential upon RET but introduces an intracellular cysteine which activate RET.
128. strong propensity to self-association in the RET-transmembrane underlies - and may be required for - dimer formation and oncogenic activation of juxtamembrane cysteine mutants of RET
129. association of high-level Ret proto-oncogene protein expression with neuronal morphology suggests tha the variable overexpression of Ret in pheochromocytomas might in part be an epiphenomenon, reflecting the known phenotypic plasticity of these tumors
130. Ret expression is significantly higher in thyroid papillary carcinoma than benign thyroid tissue; and this characteristic can have important diagnostic value.
131. association of RET IVS1-126G>T variant with sporadic medullary thyroid cancer in a cohort of 104 patients
132. mechanisms leading to RET oncogenic conversion
133. findings support the notion that both RET alleles are involved in the pathogenesis of a subgroup of Hirschsprung disease patients in a dose-dependent fashion
134. These findings establish a mechanism for the differential down-regulation of RET9 and RET51 signaling that could explain the apparently paradoxical activities of these two RET isoforms.
135. A RET haplotype (A-C-A) composed of alleles at three SNPs is associated with reduced RET gene expression in Hirschsprung patients.
136. a common non-coding RET variant within a conserved enhancer-like sequence in intron 1 is significantly associated with Hirschsprung disease susceptibility

137. Gas1 is related to the GDNF alpha receptors and regulates Ret signaling
138. We show that RA-induced differentiation is mediated by a positive autocrine loop that sustains Ret downstream signaling and depends on glial cell-derived neurotrophic factor expression and release.
139. the RET finger protein has a role in estrogen receptor-mediated transcription in tumor cells
140. A variant located in the 3' untranslated region of the RET gene, which slows down mRNA decay in patients with Hirschsprung disease.
141. study demonstrated RET amplification in all 3 cases of radiation-associated thyroid cancers(papillary thyroid cancer (PTC) & anaplastic thyroid cancer(ATC) but not in sporadic well-differentiated PTC; RET amplification was observed in all 6 cases of ATCs
142. critical role of the immunoglobulin domain in regulation of the localization of human PTPmu in bovine ce lines
143. The presence of coiled-coil domains in the ktn1/ret fusion protein (PTC8) suggests ligand-independent dimerization and thus constitutive activation of the ret tyrosine kinase domain.

## Interactions

**Description .....**

| Product | Interactant | Other Gene | Complex | Source | Pubs |
|---|---|---|---|---|---|
| RET interacts with FRS2 | | | | | |
| NP_065681.1 | NP_006645.2 | FRS2 | | BIND | PubMed |
| RET9 phosphorylates PLC-gamma via its amino-terminal SH2 domain. This interaction was modelled on a demonstrated interaction between RET9 from human and PLC-gamma from cow. | | | | | |
| NP_065681.1 | NP_002651.2 | PLCG1 | | BIND | PubMed |
| RET9 phosphorylates Shc. | | | | | |
| NP_065681.1 | NP_003020.2 | SHC1 | | BIND | PubMed |
| | | | | | |
| NP_066124.1 | NP_005179.1 | CBL | | HPRD | PubMed |
| | | | | | |
| NP_066124.1 | Docking protein 1 | DOK1 | | HPRD | PubMed |
| | | | | | |
| NP_066124.1 | DOK2 | DOK2 | | HPRD | PubMed |
| | | | | | |
| NP_066124.1 | Docking protein 3 | DOK3 | | HPRD | PubMed |
| | | | | | |

## General gene information

### Markers

**STS-T15350**(e-PCR)
Links: UniSTS:15110
Alternate names: RH40273; sts-T15350

**RH66458**(e-PCR)
Links: UniSTS:20244
Alternate name: stSG35889

**D10S1566**(e-PCR)
Links: UniSTS:23844
Alternate names: G00-588-355; GDB:588355; RH50947; UTR-03097; WI-7098

**G59921**(e-PCR)
Links: UniSTS:137202
Alternate name: SHGC-130704

**GDB:193843**(e-PCR)
Links: UniSTS:155759

**GDB:281473**(e-PCR)
Links: UniSTS:156383

**GDB:342173**(e-PCR)
Links: UniSTS:156640

**GDB:342177**(e-PCR)
Links: UniSTS:156641

**GDB:574049**(e-PCR)
Links: UniSTS:157789

**GDB:579598**(e-PCR)
Links: UniSTS:157821

**GDB:580702**(e-PCR)
Links: UniSTS:157848

**GDB:592843**(e-PCR)
Links: UniSTS:157925

**PMC23476P2**(e-PCR)
Links: UniSTS:272204

## Genotypes

See RET SNP GeneView Report
See RET SNP Genotype Report

## Phenotypes

Central hypoventilation syndrome, congenital
MIM: 209880
Colonic aganglionosis, total, with small bowel involvement
MIM: 164761
Hirschsprung disease

MIM: 142623
Medullary thyroid carcinoma
MIM: 155240
Multiple endocrine neoplasia IIA
MIM: 171400
Multiple endocrine neoplasia IIB
MIM: 162300
Pheochromocytoma
MIM: 171300

## Pathways

KEGG pathway: Thyroid cancer
05216

## Homology

Mouse, Rat
Map Viewer

## GeneOntology

Provided by GOA

| Function | Evidence |
|---|---|
| ATP binding | IEA |
| ATP binding | NAS |
| calcium ion binding | IEA |
| kinase activity | IEA |
| nucleotide binding | IEA |
| protein-tyrosine kinase activity | NAS |
| protein-tyrosine kinase activity PubMed 7824936 | TAS |
| receptor activity | IEA |
| receptor activity PubMed 7824936 | TAS |

| | |
|---|---|
| transferase activity | IEA |

| Process | Evidence |
|---|---|
| homophilic cell adhesion | IEA |
| posterior midgut development<br>PubMed 8114939 | TAS |
| protein amino acid phosphorylation | NAS |
| protein amino acid phosphorylation<br>PubMed 7824936 | TAS |
| signal transduction<br>PubMed 7824936 | TAS |

| Component | Evidence |
|---|---|
| integral to membrane | IEA |
| membrane | IEA |

## General protein information

**Names**

ret proto-oncogene
oncogene RET
Hirschsprung disease 1
receptor tyrosine kinase
RET transforming sequence
cadherin family member 12
hydroxyaryl-protein kinase
multiple endocrine neoplasia and medullary thyroid carcinoma 1
ret proto-oncogene (multiple endocrine neoplasia and medullary thyroid carcinoma 1, Hirschsprung disease)

**NP_065681.1**

EC 2.7.10.1

**NP_066124.1**
EC 2.7.10.1

## NCBI Reference Sequences (RefSeq)

### RefSeqs maintained independently of Annotated Genomes

These reference sequences exist independently of genome builds.

***mRNA and Protein(s)***

1. **NM_020630.4→NP_065681.1 ret proto-oncogene isoform c**

| | |
|---|---|
| **Description** | Transcript Variant: This variant (4) differs in the 3' UTR and coding region compared to variant 2. The resulting isoform (c) is shorter and has a distinct C-terminus compared to isoform a. This isoform is also known as Ret9. |
| **Source sequence(s)** | AI472270,BC003072,BC004257,BE261914,BM703293,DA100452,DA911581,X12949 |

**Conserved Domains (10) summary**

| | | |
|---|---|---|
| | **cd00031**<br>**Location:173–358**<br>**Blast Score:188**<br>**Location:178–302**<br>**Blast Score:188**<br>**Location:43–264**<br>**Blast Score:106**<br>**Location:43–245**<br>**Blast Score:106** | CA; Cadherin repeat domain; Cadherins are glycoproteins involved in Ca2+-mediated cell-cell adhesion; these domains occur as repeats in the extracellular regions which are thought to mediate cell-cell contact when bound to calcium; plays a role in cell fate, |
| | **cd00192**<br>**Location:716–1008**<br>**Blast Score:971**<br>**Location:872–881**<br>**Blast Score:971**<br>**Location:788–893**<br>**Blast Score:971**<br>**Location:892–921**<br>**Blast Score:971**<br>**Location:813–915**<br>**Blast Score:971** | TyrKc; Tyrosine kinase, catalytic domain. Phosphotransferases; tyrosine-specific kinase subfamily. |
| | **smart00220**<br>**Location:726–1004**<br>**Blast Score:449** | S_TKc; Serine/Threonine protein kinases, catalytic domain; Phosphotransferases. Serine or threonine-specific kinase subfamily. |

2. **NM_020975.4→NP_066124.1 ret proto-oncogene isoform a**

| | |
|---|---|
| **Description** | Transcript Variant: This variant (2) represents the longer transcript and encodes the longer isoform (a). This isoform is also known as Ret51. |
| **Source sequence(s)** | AC010864,BC003072,BC004257,BM661773,DA100452,X12949 |
| **Consensus CDS** | CCDS7200.1 |

**Conserved Domains (10) summary**

| | |
|---|---|
| **cd00031**<br>**Location:173–358**<br>**Blast Score:188**<br>**Location:178–302**<br>**Blast Score:188**<br>**Location:43–264**<br>**Blast Score:105**<br>**Location:43–245**<br>**Blast Score:105** | CA; Cadherin repeat domain; Cadherins are glycoproteins involved in Ca2+-mediated cell-cell adhesion; these domains occur as repeats in the extracellular regions which are thought to mediate cell-cell contact when bound to calcium; plays a role in cell fate, |
| **cd00192**<br>**Location:716–1008**<br>**Blast Score:973**<br>**Location:872–881**<br>**Blast Score:973**<br>**Location:788–893**<br>**Blast Score:973**<br>**Location:892–921**<br>**Blast Score:973**<br>**Location:813–915**<br>**Blast Score:973** | TyrKc; Tyrosine kinase, catalytic domain. Phosphotransferases; tyrosine-specific kinase subfamily. |
| **smart00220**<br>**Location:726–1004**<br>**Blast Score:451** | S_TKc; Serine/Threonine protein kinases, catalytic domain; Phosphotransferases. Serine or threonine-specific kinase subfamily. |

## RefSeqs of Annotated Genomes: Build 36.2

The following sections contain reference sequences that belong to a specific genome build.

### Reference assembly

***Genomic***

1. **NC_000010.9 Reference assembly**

| | |
|---|---|
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### Alternate assembly (based on Celera assembly)

***Genomic***

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The following Reference Sequences have been suppressed. Explain

1. **NM_000323.2: Suppressed sequence**

| | |
|---|---|
| **Description** | NM_000323.2 was permanently suppressed because currently not enough support exists for the transcript and the protein. |

2. **NM_020629.2: Suppressed sequence**

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| **Description** | NM_020629.2 was permanently suppressed because currently not enough support exists for the transcript and the protein. |

## Related Sequences

| Nucleotide | | Protein |
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| Genomic | AF520975.1 | AAM77275.1 |
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| Genomic | AJ243297.1 | CAB46483.1 |
| Genomic | CH471160.1 | EAW86576.1 |
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| | | EAW86578.1 |
| | | EAW86579.1 |
| | | EAW86580.1 |

| | | |
|---|---|---|
| | | EAW86581.1 |
| | | EAW86582.1 |
| | | EAW86583.1 |
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| Genomic | Y15743.1 | CAA75753.1 |
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| mRNA | AW297789.1 | None |
| mRNA | BC003072.2 | AAH03072.1 |
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| mRNA | BE261914.1 | None |
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| mRNA | BM703293.1 | None |
| mRNA | BQ070075.1 | None |
| mRNA | BX332519.2 | None |
| mRNA | BX376396.2 | None |
| mRNA | DA100452.1 | None |
| mRNA | DA911581.1 | None |
| mRNA | H24956.1 | None |
| mRNA | X15262.1 | CAA33333.1 |
| mRNA | Y12528.1 | CAA73131.1 |

| Protein Accession | Links | |
|---|---|---|
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| Q15850 | GenPept | UniProtKB/TrEMBL |
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| Q9UMQ4 | GenPept | UniProtKB/TrEMBL |
| Q9UQV8 | GenPept | UniProtKB/TrEMBL |

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# CANCER
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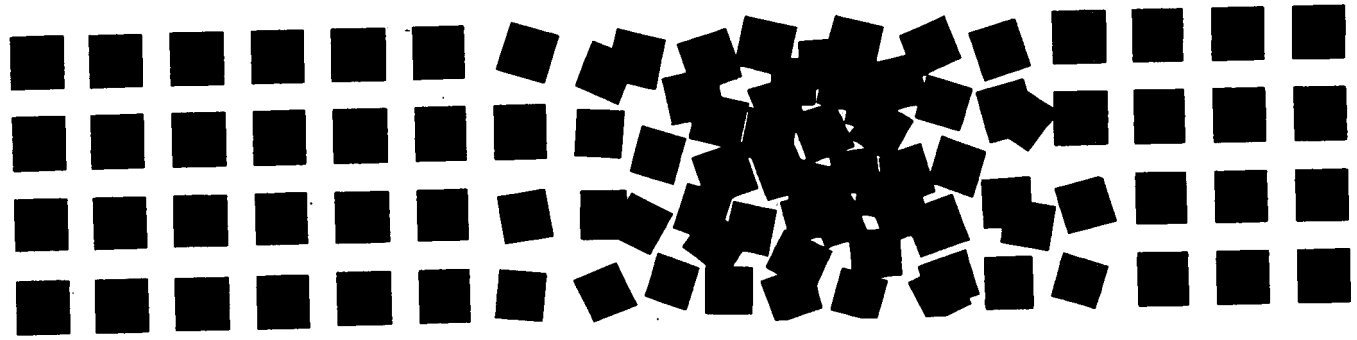

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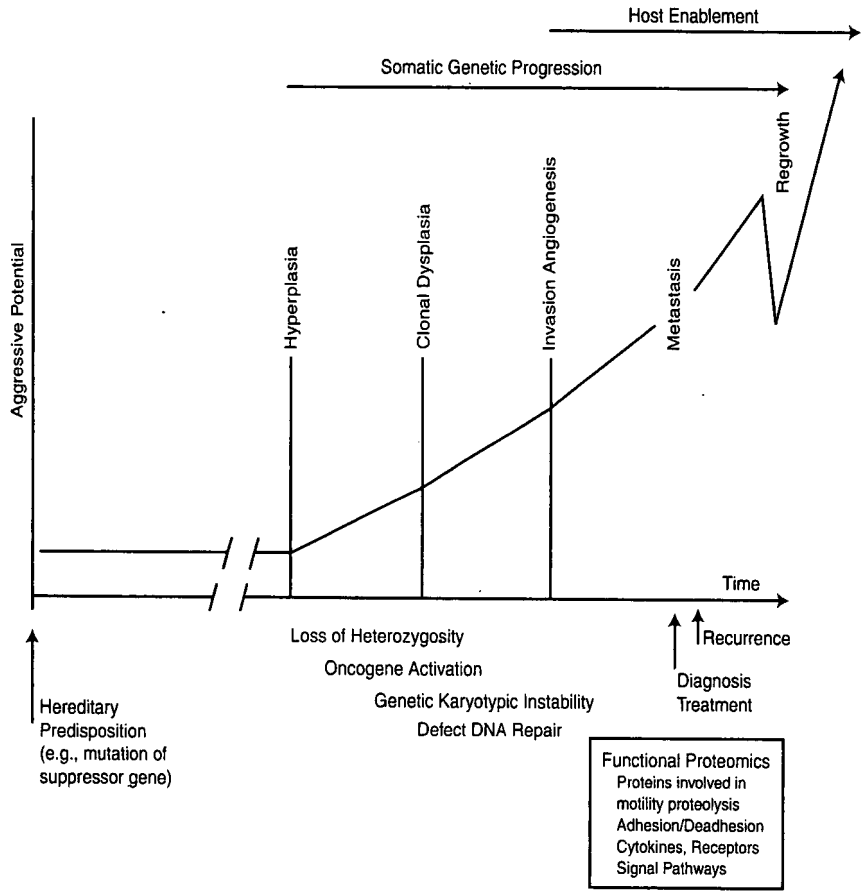


**FIGURE 2-1.** Molecular progression of cancer. Microscopic premalignant lesions originate within a background of hereditary predisposition. Genetic instability leads to molecular derangements that drive somatic genetic progression. Host interactions enable the cancer lesions to expand, vascularize, and metastasize. Diagnosis usually takes place at a late stage when there is a high probability of metastasis.

Separate genetic changes (beyond those causing uncontrolled growth) are required for tumor invasion and metastasis. Invasion and metastasis form a multistep cascade involving positive and negative regulatory pathways. Cancer invasion and angiogenesis are an uncontrolled version of physiologic invasion.

Genetic instability may predispose the premalignant cell to generate malignant offspring. Instability can take place at the macro level (chromosome karyotype), as well as the micro level (DNA sequence copy fidelity repair). Chromosomal rearrangement can activate silent oncogenes or delete regions containing suppressor genes. Loss of heterozygosity is a hallmark of suppressor gene inactivation in cancer progression. Telomerase defects may affect growth control as well as genetic instability. Mutations in cellular DNA can activate oncogenes or inactivate suppressor genes. Defects in DNA repair mechanisms contribute to the accumulation of genetic defects fueling cancer progression. Genetic defects causing an inhibition of cell death pathways are an important mechanism in tumorigenesis.

## CANCER GENES: MODELS OF ACTION

Genetic alterations involved in cancer can activate inductive processes (oncogenes) or block negative pathways (suppressor genes). Early models of cancer genetics categorized cancer genes into oncogenes, which are growth inducing, and tumor suppressor genes, which are growth suppressing. Thus, mutations in oncogenes activate a promoting function, but lesions in tumor suppressors inactivate an inhibitory function. Examples of these models were the ras oncogene and the retinoblastoma (rb) tumor suppressor gene. Mutations in codons 12, 13, and 61 in the ras gene result in biochemical activation of the protein product and an induction of its transforming activity. Deletions or inactivating mutations in the rb gene lead to a compromised suppressor protein that is incapable of inhibiting cell growth. Aberrations in both genes are found as somatic mutations in human cancers and, in the case of rb, also in the germline of individuals at risk for cancer.

Dominant oncogenes play a significant role in human cancers. Ras mutations are found in 10% of cancers and appear frequently in colon and lung adenocarcinomas. Ret is a receptor tyrosine kinase in which activating single nucleotide mutations are associated with hereditary thyroid carcinomas. Myc, encoding a nuclear oncoprotein, is involved in the t(8;14) translocation, which is etiologic for Burkitt's lymphoma. Inappropriate overexpression of myc is sufficient for transformation of lymphocytes in transgenic models. Similarly, amplification and overexpression of the HER2/neu receptor tyrosine kinase not only causes mammary malignancies, but is prognostic in human breast cancers. Although originally these oncogene abnormalities were thought to induce cancer primarily through unregulated growth, other cellular phenotypes such as enhanced survival and motility may be equally important contributors to the cancer state.

It was also originally thought that tumor suppressor genes function mainly by inhibiting cell growth. Later, this was expanded to genes that block the emergence of a tumor, but not growth in culture. More recent studies, however, have uncovered other mechanisms unrelated to growth by which *tumor suppressor*

**FIGURE 38.1-3.** Photomicrograph of medullary thyroid carcinoma showing nests and sheets of small, uniform cells with scant to moderate amounts of amphophilic cytoplasm infiltrating around normal thyroid follicles. (Reprinted from Moley JF, Lairmore TC, Phay JE, Hereditary endocrinopathies. *Curr Probl Surg* 1999;36:653, with permission.)

In MEN 2B, 40% to 50% of patients develop pheochromocytomas, and all individuals develop neural gangliomas, particularly in the mucosa of the digestive tract, conjunctiva, lips, and tongue. MEN 2B patients also have megacolon, skeletal abnormalities, and markedly enlarged peripheral nerves. MEN 2B patients do not develop HPT. MTC develops at a very young age (infancy) and appears to be the most aggressive form of hereditary MTC, although its aggressiveness may be related more to the extremely early age of onset than to the biologic virulence of the tumor. MTC in MEN 2B is rarely curable.

FMTC is characterized by the development of MTC without any other endocrinopathies.[26] MTC in these patients has a later age of onset and follows a more indolent clinical course than MTC in patients with MEN 2A and MEN 2B. Occasional patients with FMTC never manifest clinical evidence of MTC (symptoms or a lump in the neck), though biochemical testing and histologic evaluation of the thyroid usually demonstrates MTC.

## Genetics

THE *RET* PROTOONCOGENE. In 1987, the gene for MEN 2A was localized to the pericentromeric region of chromosome 10 (10q11.2) by linkage analysis.[27,28] Subsequent studies demonstrated that the predisposition gene for MEN 2B and FMTC mapped to the same region as MEN 2A.[29,30] The *RET* protooncogene resides within this critical region, which made it an obvious candidate gene for the MEN 2 syndromes.

The *RET* protooncogene was first discovered based on its ability to transform mouse NIH 3T3 fibroblasts in culture.[31] The transforming *RET* sequences first identified represented a rearrangement of *RET* that occurred *in vitro* during the transfection assay.[32] Sequence analysis of the *RET* protooncogene showed that it is a member of the receptor tyrosine kinase gene family.[33] *RET* was mapped to the proximal region of the long arm of chromosome 10 in 1989[34] and was shown to be expressed at high levels in both MTCs and pheochromocytomas.[35]

*RET* mutations were identified in the constitutional DNA of MEN 2A and FMTC patients in 1993.[36,37] Mulligan et al.[36] identified *RET* mutations in association with MEN 2 using an expression-based mutational analysis system. Analysis of genomic DNA from MEN 2A family members proved that the variants identified in the complementary DNAs (cDNAs) were also in the constitutional DNA of affected family members and not in individuals unaffected by MEN 2A. All mutations resulted in substitution of cysteine residues clustered near the transmembrane domain of *RET*. In all but one, MEN 2A tumor heterozygosity for the mutant and wild-type *RET* allele was retained. Mulligan et al.[36] suggested a dominant or dominant-negative mechanism for *RET* mutation in the development of MTC and pheochromocytomas in MEN 2A.[36]

Donis-Keller et al.[37] used single-strand conformational variant (SSCV) and sequence analysis to identify mutations in *RET* in the constitutional DNA of patients with MEN 2A and FMTC. Both MEN 2A and FMTC were shown to be associated with mutations that result in substitution of cysteine residues in the extracellular portion of *RET* immediately adjacent to the transmembrane domain. Unexpectedly, the same mutations were found to characterize MEN 2A and FMTC kindreds.

Subsequent investigations demonstrated mutations of *RET* in MEN 2B patients.[38,39] In 95% of cases of MEN 2B, the *RET* protooncogene mutation is a missense methionine-to-threonine (ATG to ACG) change at codon 918 in exon 16. This codon is positioned within the tyrosine kinase catalytic core of the intracellular domain. Two families with MEN 2B have been described that have a codon 883 mutation in the tyrosine kinase domain of *RET*.[40]

More than 30 missense mutations have been described in MEN 2A and FMTC kindreds (Table 38.1-2).[41,42] Most of these mutations result in nonconservative changes in cysteine residues, although changes in Glu, Val, Met, Leu, and Tyr have also been described. Several of these mutations have been shown to result in "gain-of-function" in the *RET* protein product, with increased intrinsic tyrosine kinase activity or alterations of substrate recognition (or both) and transforming capability.[43] The *RET* protooncogene encodes a protein with three domains: a cysteine-rich extracellular receptor domain, a hydrophobic transmembrane domain, and an intracellular tyrosine kinase catalytic domain (Fig. 38.1-4). The *RET* gene consists of at least 20 exons[44] and is expressed as five major mRNA species.[45,46] The *RET* gene product is expressed in a limited number of cell types in the normal individual, including the thyroid C cells, the adrenal medulla, and parts of the brain. The gene is important in the embryonic development of the enteric nervous system and the kidneys.[47]

**TABLE 38.1-2.** *RET* Mutations in Hereditary Medullary Thyroid Carcinoma

| | *Missense Germline Mutations in the RET Protooncogene* | |
|---|---|---|
| *Syndrome* | Exon | Codon |
| MEN 2A, FMTC | 10 | 609 |
| | | 611 |
| | | 618 |
| | | 620 |
| | 11 | 631[a] |
| | | 634 |
| | 13 | 790 |
| | | 791 |
| FMTC | 11 | 630 |
| | 13 | 768 |
| | 14 | 804 |
| | | 844[a] |
| | 15 | 891 |
| MEN 2B | 16 | 918 |
| | | 883 |

FMTC, familial, non-MEN medullary thyroid carcinoma; MEN 2A, multiple endocrine neoplasia type 2A; MEN 2B, multiple endocrine neoplasia type 2B.
[a]Clinical features not yet characterized.
(Reprinted from Moley JF, Lairmore TC, Phay JE. Hereditary endocrinopathies. *Curr Probl Surg* 1999;36:653, with permission.)

Glial-derived neurotrophic factor (GDNF) and neurturin are ligands to the receptor domain of the *RET* gene product.[48–54] GDNF is a 32-kD protein dimer that was first purified from glial cell lines and is a potent neurotrophic survival factor for motor neurons. A glycophosphatidylinositol-linked protein called *GDNF receptor-a* (GDNFR-α) is a cofactor in the signaling heterodimeric complex with *RET*. Current evidence suggests that GDNF binds directly to GDNFR-α and indirectly with *RET*. When triggered by ligand, wild-type *RET* dimerizes with another *RET* molecule, and this dimerization is responsible for phosphorylation and activation of the tyrosine kinase domain, with subsequent downstream signal transduction events. *RET* molecules that contain MEN 2A-type mutations are constitutively dimerized and therefore activated. In contrast, the mutation responsible for MEN 2B does not result in constitutive dimerization but changes the substrate specificity of the tyrosine kinase domain, which results in transformation.

OTHER *RET* GENOTYPE-PHENOTYPE CORRELATIONS

*Cutaneous Lichen Amyloidosis.* Interscapular lesions of cutaneous lichen amyloidosis (CLA) have been described in several families with MEN 2A.[22] The total number of patients described with this entity is fewer than 100. In a 634→Tyr mutation was reported in one family with MEN 2A and CLA, and in two other families, a 634→Arg mutation was described that segregated with both *MEN2A* and CLA.[55]

*Hyperparathyroidism.* HPT in MEN 2A is caused by parathyroid hyperplasia; the hypercalcemia is mild and often asymptomatic. HPT clusters in some families with MEN 2A. Whether specific *MEN2A* mutations are associated with a higher incidence of HPT remains controversial. Mulligan et al.[56] previously described a strong correlation between C634R mutation and HPT in families with MEN 2A, but other studies have been unable to confirm this relationship definitively.[57–59]

*Hirschsprung's Disease.* Hirschsprung's disease is characterized by absence of autonomic ganglion cells within the distal colonic parasympathetic plexus, resulting in obstruction and proximal megacolon. Approximately 80% of Hirschsprung's disease cases are sporadic, and the remainder are familial. A subset of familial Hirschsprung's cases have been found to be associated with germline mutations of *RET*.[24,56] Most of these are inactivating and loss-

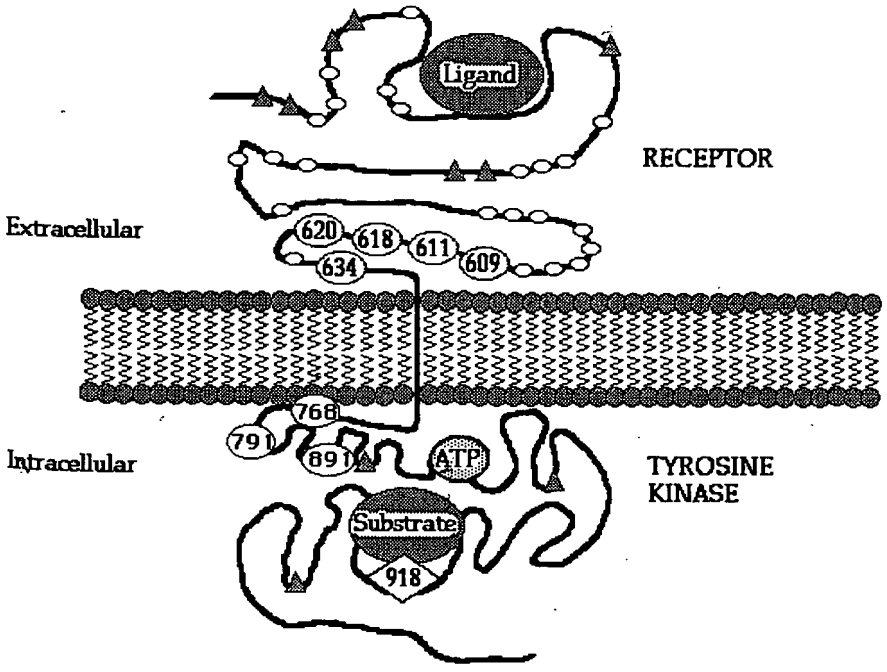


**FIGURE 38.1-4.** Diagram of *RET* gene product delineating locations of germline mutations found in multiple endocrine neoplasia type 2A and familial, non-MEN medullary thyroid carcinoma (*ovals*), germline mutations in multiple endocrine neoplasia type 2B (*diamond*), and mutations in hereditary Hirschsprung's disease (*triangles*). As mentioned in the text, the *RET* gene product is thought to create a dimer that forms a complex with glial-derived neurotrophic factor receptor-α or neurturin. The *RET* gene product is divided into the intracellular, transmembrane, and extracellular domains. ATP, adenosine triphosphate. (Adapted from Moley JF, Kim S, *Molecular genetics in surgical oncology*. Austin: RG Landes, 1994, with permission.)

**TABLE 38.1-3.** Loss of Heterozygosity in Pheochromocytomas and Medullary Thyroid Carcinomas

| *Study* | *Chromosomal Arms Tested* | *LOH in Pheos (No. LOH/No. Informative)* | *LOH in MTCs (No. LOH/No. Informative)* |
|---|---|---|---|
| Khosla et al.[178] | 1p, 2p, 3p, 5q, 10q, 13q, 16p, 17p, 17q, 22q | 1p-13/31, 2p-1/34, 3p-4/24, 10q-1/22, 17p-7/27, 22q-5/18 | 1p-1/11, 22-1/7 |
| Moley et al.[69] | 1p, 1q | 1p-12/18 (9/9 from MEN 2A and 2B patients) | 1p-3/24 |
| Yang et al.[179] | 1p | 1p-5/8 | |
| Mathew et al.[180] | 1p, 1q, 5, 6, 7, 11, 12 | 1p-4/6 | 1p-3/8 |
| Shin et al.[181] | 1p, 22q | 1p-12/22, 22q-8/20 | |
| Mulligan et al.[70] | 1-22, both arms | 1p-15/25, 3p-10/18, 3q-9/15, 5q-1/7, 6q-1/7, 8p-1/8, 11p-3/19, 11q-2/23, 13-2/17, 17p-3/20, 17q-1/20, 22-8/20 | 1p-7/28, 3p-1/19, 3q-2/14, 7p-1/17, 10p-1/18, 10q-1/25, 11p-1/16, 13-2/22, 15-1/9, 21-1/8, 22-4/22 |
| Dou et al.[73] | 2-23 | 3q-13/13 F, 6/8 S, 21q-4/6 F, 2/7 S, 22q-7/13 F, 1/10 S, 11q-2/6 F, 3/7 S | 3q-2/7 F, 1/8 S, 22q-4/15 F, 2/8 S |
| Herfarth et al.[74] | 17p | 17p-4/22 | 17p-0/14 |

F, familial (MEN 2A and 2B); LOH, loss of heterozygosity; MEN 2A, multiple endocrine neoplasia type 2A; MEN 2B, multiple endocrine neoplasia type 2B; MTCs, medullary thyroid carcinomas; Pheos, pheochromocytomas; S, sporadic (MEN 2A and 2B).
Note: If arm tested is not listed in LOH column, LOH was not found.
(Adapted from J Moley, Molecular events in the development and progression of medullary thyroid cancer and pheochromocytoma. In: Nelkin B, ed., *Genetic mechanisms in multiple endocrine neoplasia type 2.* Austin: RG Landes, 1996, with permission.)

of-function (frameshift and nonsense) mutations and are not associated with the MEN 2A phenotype. Several families, however, have been described in which Hirschsprung's disease cosegregates with either *MEN2A* or *FMTC* (missense codon 618 or 620 mutations).[60] Additionally, a few Hirschsprung's disease patients have been described with missense mutations in codon 609 or 620 who have no evidence of MEN 2A or MTC.[23,25,56] It is interesting to note that the Hirschsprung's disease phenotype can be associated with either loss-of-function or gain-of-function mutations of *RET*. All patients with MEN 2B (missense codon 918 mutation) have megacolon and chronic colonic motility disturbances, though they usually do not require surgery for this (see Fig. 38.1-2*C*).[61]

*RET Mutations in Sporadic Tumors.* Mutations in the *RET* protooncogene have also been found in sporadic MTCs.[37,40,62–64] The most frequent mutation in sporadic MTCs is the M918T mutation found in MEN 2B. Mutations have been found in other regions of the extracellular and intracellular domains. Missense, deletions, and insertion mutations have been described.[41,65] Somatic *RET* mutations in sporadic pheochromocytomas are unusual but have also been described.[41]

*Other Dominant Oncogenes in Medullary Thyroid Carcinomas and Pheochromocytomas.* Absence of amplification of *N-MYC, C-MYC,* and *ERB-B2* has been reported in MTCs and pheochromocytomas. Roncalli et al.[66] reported that N-myc expression in more than 10% of tumor cells, as detected by immunohistochemistry, was associated with poorer survival, sporadic disease, and male gender. These investigators found no evidence of gene amplification and did not determine the basis for the overexpression.[66] Our group reported absence of mutation of the H-*RAS,* N-*RAS,* and K-*RAS* genes in a series of pheochromocytomas and MTCs analyzed by direct sequencing.[67] Likewise, examination of nerve growth factor and nerve growth factor receptor (p75) showed no abnormality at the DNA or RNA levels.[68]

*Other Tumor Suppressor Genes in Medullary Thyroid Carcinoma and Pheochromocytoma.* Several studies have evaluated loss of heterozygosity (LOH) at tumor suppressor loci in pheochromocytomas and MTCs; these are summarized in Table 38.1-3. The cumulative data indicate a higher-than-background incidence of LOH in pheochromocytomas on chromosome arms 1p, 3p, 17p, and 22q.[69,70] In MTCs, the report by Mulligan et al.[70] suggests a significant incidence of 1p LOH; however, evaluation of other chromosomal arms yielded no consistent findings. Lack of significant LOH on 10q, at the *RET* locus, supports the hypothesis that the *RET* protooncogene acts as a dominant oncogene as opposed to a tumor suppressor gene.[71] Chromosome 1 is the largest chromosome, and LOH analysis on 1p in pheochromocytomas suggests a very large region of deletion. Our studies have indicated that the entire short arm of 1p is lost in pheochromocytomas from patients with MEN 2A and MEN 2B.[69] Fine mapping of the region of deletion suggests a possible common breakpoint in the centromeric region defined by the markers D1S514 and D1S442.[72]

The high rate of LOH on 3p in pheochromocytomas suggests an as-yet undefined tumor suppressor locus.[70,73] LOH on 17p suggests possible involvement of the *TP53* gene.[74] Existing reports on *TP53* mutations in pheochromocytomas are conflicting. Two Japanese groups reported no evidence of *TP53* mutations in pheochromocytomas.[75,76] In contrast, a Chinese group reported *TP53* mutations in five of six tumors tested.[77] Four of these mutations were in exon 4. Our group reported a series of 22 pheochromocytomas and 29 MTCs that were screened with four different markers for LOH on 17p.[74] SSCV analysis of exons 4 through 9 of the *TP53* gene was performed in 20 of the pheochromocytomas and in 22 of the MTCs. The expression of p53 was determined by immunohistochemistry in 19 pheochromocytomas and in 17 MTCs, using two different antibodies (D01 and D07) on both frozen and paraffin-embedded tissues. LOH was demonstrated on 17p in 4 of the 22 pheochromocytomas and in none of the MTCs. No mutations were detected in any of the tumors screened by SSCV analysis. Immunohistochemical staining of frozen and paraffin-embedded tumor sections did not show p53 overexpression in any of the tumors examined. These findings indicate that mutations in the *TP53* gene are an uncommon event in the tumorigenesis of hereditary and sporadic pheochromocytomas and MTCs.[74]

Pheochromocytomas also occur in neurofibromatosis type 1 (NF1) and von Hippel-Lindau (VHL) disease, both of which

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**EXHIBIT G**

# RET proto-oncogene

From Wikipedia, the free encyclopedia

The ***RET* proto-oncogene** encodes a receptor tyrosine kinase for members of the glial cell line-derived neurotrophic factor family of extracellular signalling molecules.[1] *RET* loss of function mutations are associated with the development of Hirschsprung's disease, while gain of function mutations are associated with the development of various types of human cancer, including medullar thyroid carcinoma and multiple endocrine neoplasias type 2A and type 2B.

| ret proto-oncogene | |
|---|---|
| **Identifiers** | |
| Symbol | RET |
| Alt. Symbols | HSCR1, MEN2A, MTC1, MEN2B |
| Entrez | 5979 (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene&cmd=retrieve&dopt=default&list_uids=5979&rn=1) |
| HUGO | 9967 (http://www.genenames.org/data/hgnc_data.php?hgnc_id=9967) |
| OMIM | 164761 (http://www.ncbi.nlm.nih.gov/entrez/dispomim.cgi?id=164761&rn=1) |
| RefSeq | NM_020975 (http://genome.cse.ucsc.edu/cgi-bin/hgGene?org=Human&hgg_gene=NM_020975&rn=1) |
| UniProt | P07949 (http://www.expasy.org/cgi-bin/niceprot.pl?P07949) |
| **Other data** | |
| Locus | Chr. 10 *q11.2* *(http://www.ncbi.nlm.nih.gov/Omim/getmap.cgi?chromosome=10q11.2)* |

## Contents



## Structure

*RET* is an abbreviation for "rearranged during transfection", as the DNA sequence of this gene was originally found to be rearranged within a 3T3 fibroblast cell line following its transfection with DNA taken from human lymphoma cells.[2] The human gene *RET* is localized to chromosome 10 (10q11.2) and contains 21 exons.[3]

The natural alternate splicing of the *RET* gene results in the production of 3 different isoforms of the protein RET. RET51, RET43 and RET9 contain 51, 43 and 9 amino acids in their C-terminal tail respectively.[4] The biological roles of isoforms RET51 and RET9 are the most well studied *in-vivo* as these are the most common isoforms in which RET occurs.

Common to each isoform is a domain structure. Each protein is divided into three domains: an N-terminal extracellular domain with four

cadherin-like repeats and a cysteine-rich region, a hydrophobic transmembrane domain and a cytoplasmic tyrosine kinase domain, which is split by an insertion of 27 amino acids. Within the cytoplasmic tyrosine kinase domain, there are 16 tyrosines (Tyrs) in RET9 and 18 in RET51. Tyr1090 and Tyr1096 are present only in the RET51 isoform.[5]

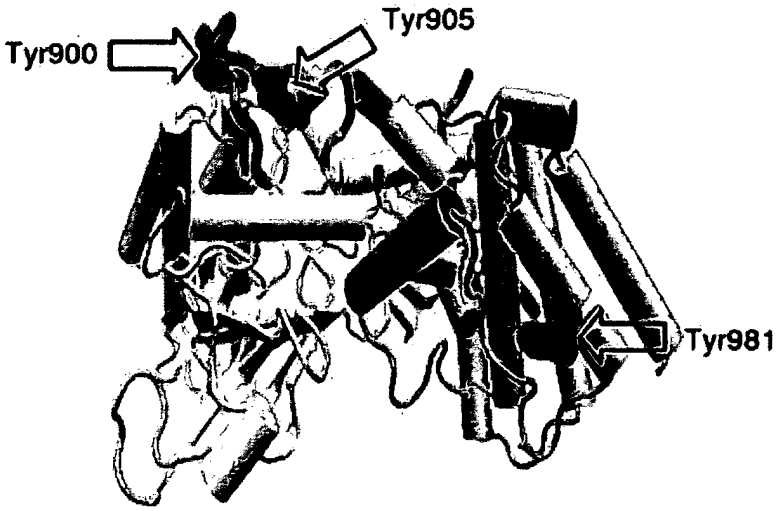
The extracellular domain of RET contains nine N-glycosylation sites. The fully glycosylated RET protein is reported to have a molecular weight of 170 kDa although it is not clear to which isoform this molecular weight relates.[6]

## Kinase activation

RET is the receptor for members of the glial cell line-derived neurotrophic factor (GDNF) family of extracellular signalling molecules or ligands (GFLs).[7]

In order to activate RET GFLs first need to form a complex with a glycosylphosphatidylinositol (GPI)-anchored co-receptor. The co-receptors themselves are classified as members of the GDNF receptor-α (GFRα) protein family. Different members of the GFRα family (GFRα1-GFRα4) exhibit a specific binding activity for a specific GFLs.[8] Upon GFL-GFRα complex formation, the complex then brings together two molecules of RET, triggering trans-autophosphorylation of specific tyrosine residues within the tyrosine kinase domain of each RET molecule. Tyr900 and Tyr905 within the activation loop (A-loop) of the kinase domain have been shown to be autophosphorylation sites by mass spectrometry.[9] Phosphorylation of Tyr905 stabilizes the active conformation of the kinase which in turn results in the autophosphorylation of other tyrosine residues mainly located in the C-terminal tail region of the molecule.[5]

The structure shown below was taken from the protein data bank code 2IVT (http://www.pdb.org/pdb/explore.do?structureId=2IVT).[1] The structure is that of a dimer formed between two protein molecules each spanning from amino acids 703-1012 of the RET molecule, covering RETs intracellular tyrosine kinase domain. One protein molecule, molecule A is shown in yellow and the other, molecule B in grey. The activation loop is coloured purple and selected tyrosine residues in green. Part of the activation loop from molecule B is absent.




Phosphorylation of Tyr981 and the additional tyrosines Tyr1015, Tyr1062 and Tyr1096 not covered by the above structure, have been shown to be important to the initiation of intracellular signal transduction processes.

## Role of RET signalling during development

Mice deficient in GDNF, GFRα1 or the RET protein itself exhibit severe defects in kidney and enteric nervous system development. This implicates RET signal transduction as key to the development of normal kidneys and the enteric nervous system.[5]

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## External links

- MeSH *ret+Proto-Oncogene+Proteins (http://www.nlm.nih.gov/cgi/mesh/2007/MB_cgi?mode=&term=ret+Proto-Oncogene+Proteins)*

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